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Intracellular calcium and growth hormone releasing hormone (GHRH)-stimulated adenosine 3'5'-monophosphate (cAMP) accumulation in anterior pituitary cells

By

Donald J. Hartt

A thesis submitted to the Faculty of Graduate Studies and Research Fulfillment
in partial fulfilment of the requirements for the degree of
Master of Science

Department of Physiology

Edmonton, Alberta

Fall, 1995

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Title of thesis: **Intracellular calcium and growth-hormone releasing hormone (GHRH)-stimulated adenosine 3'5'-monophosphate (cAMP) accumulation in anterior pituitary cells**

Degree: **Masters of Science**

Year this degree granted: **1995**

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Abstract

In this study, the effect of thapsigargin (Tg), an intracellular Ca^{2+}-mobilizing agent, on growth hormone-releasing hormone (GHRH)-stimulated cAMP accumulation in rat anterior pituitary cells was investigated. It was found that treatment with GHRH (0.1 μM) stimulated cAMP accumulation 55-fold in dispersed anterior pituitary cells. Concurrent treatment with Tg (20 μM) further increased the GHRH-stimulated cAMP accumulation 2-fold. This effect of Tg was concentration-dependent, persisted in the presence of a phosphodiesterase inhibitor, isobutylmethylxanthine (1 mM), and was not affected by the duration of pretreatment (0 - 150 min). A similar effect of Tg was observed when the pituitary cells were stimulated with pituitary adenylate cyclase-activating polypeptide (0.1 μM), forskolin (1 μM), or cholera toxin (20 μg/ml). The potentiating effect of Tg was also observed in the pituitary-derived GH_3 tumour cell line on cholera toxin- and forskolin-stimulated cAMP accumulation. Pretreatment with BAPTA-AM (0.1 mM) and EGTA (0.5 mM) blocked the potentiating effect of Tg on agonist-stimulated cAMP accumulation, indicating that this effect is dependent on elevation of intracellular Ca^{2+}. Studies on the involvement of protein kinases showed that the effect of Tg was blocked by W7 (75 μM), but not by H7 (0.1 mM) or calphostin C (1 μM). Therefore, a Ca^{2+}/calmodulin dependent protein kinase rather than protein kinase C appears to be involved in the action of Tg. These observations suggest that Tg enhances GHRH-stimulated cAMP accumulation by increasing the rate of cAMP synthesis through activation of a Ca^{2+}/calmodulin dependent

adenylate cyclase. Also, to determine if the potentiating effect of Tg on the GHRH-stimulated cAMP accumulation was comparable to other Ca^{2+} elevating agents, a depolarizing concentration of K^+ (30 mM) was used. However, it was observed that K^+ did not have an increasing effect on the GHRH-stimulated cAMP accumulation. Instead, K^+ (30 mM) inhibited the GHRH-stimulated cAMP response. This suggests that K^+ and Tg affect the GHRH-stimulated cAMP pathway differently through different mechanisms, possibly due to the level of intracellular Ca^{2+} mobilized by each agent, which may in turn activate different adenylate cyclase isoforms or Ca^{2+}-dependent phosphodiesterases.

Acknowledgement

I would like to express my appreciation to Dr. Chik and Dr. Ho for their patience, wisdom, and friendship over the past two years. I would also like to thank Gord Murdoch, Mark Nutley, Dr. Ed Karpinski, Toshi Inukai, Marc Girard, Reid Weist, Takayuki Ogiwara, and the rest of my lab mates for their friendship and support.

The encouragement, knowledge, and guidance provided by all the members of my supervisory committee is also appreciated. I would like to thank the Medical Research Council and the department of Physiology for financial support.

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List of Abbreviations

AC:	Adenylate cyclase
BAPTA:	1,2 bis (2-Aminophenoxy) ethane-N,N,N',N'-tetraacetic Acid
BSA:	Bovine serum albumin
Ca^{2+}:	Calcium ion
$[Ca^{2+}]i$:	Intracellular calcium
CaM:	Calmodulin
cAMP:	Adenosine 3'5' monophosphate
CN:	Caudate nucleus
CRB:	Cerebellum
DG:	Dentate gyrus
DMEM:	Dulbecco's modified eagle medium
DMSO:	Dimethyl sulfoxide
EGTA:	Ethyleneglycol-bis-(B-aminoethyl ether)-N,N'-tetraacetic acid
ER:	Endoplasmic reticulum
FCS:	Fetal calf serum
FSK:	Forskolin
g:	Gram
GH:	Growth hormone
GHRH:	Growth hormone releasing hormone
Gs:	Guanine nucleotide binding protein
h:	Hour

H7:	1-(5-isoquinolinesulfonyl)-2-methylpiperazine
HEPES:	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
H:	Hippocampus
IBMX:	3-isobutyl-1-methylxanthine
IP3:	Inositol phosphate
K^+:	Potassium ion
M:	Molar
MSH:	Melanin stimulating hormone
μg:	Microgram
μl:	Microliter
μM:	Micromolar
mCi:	Millicurie (37 megaBq)
mg:	Milligram
min:	Minute
ml:	Milliliter
mM:	Millimolar
nm:	Nanometer
nM:	Nanomolar
OE:	Olfactory neuroepithelium
PACAP:	Pituitary adenylate cyclase-activating polypeptide 1-38
PBS:	Phosphate-buffered saline
PDE:	Phosphodiesterase
PIP2:	Phosphatidylinositol

I. Introduction

Intracellular Ca^{2+} ($[Ca^{2+}]i$), in addition to having a direct role in the release of growth hormone in anterior pituitary somatotrophs (Frohman et al., 1986), has also been shown to interact directly with the synthesis of cAMP (Brazeau et al., 1982). Depending on the cell type, elevation of $[Ca^{2+}]i$ can either increase or decrease agonist-stimulated cAMP accumulation. For example, in rat pinealocytes (Chik et al., 1988), human colonic cells (HT29-cl. 19A) (Warhurst et al., 1994), and rat renal papillary collecting tubule cells (Ishikawa et al., 1992), elevation of $[Ca^{2+}]i$ potentiates the agonist-stimulated cAMP accumulation, while in cardiac myocytes (Yu et al., 1993) and C_6-glioma cells (Lin et al., 1993), elevation of $[Ca^{2+}]i$ has an inhibitory effect. This suggests that the interaction between cAMP accumulation and $[Ca^{2+}]i$ is complex, and may involve multiple sites and mechanisms (Abou-Samra et al., 1987). Apart from the reported direct effect of Ca^{2+} on the adenylate cyclase activity (Amiranoff et al., 1983; Caldwell et al., 1992), and phosphodiesterase activity (Meeker et al., 1982; Van Sande et al., 1979), other possible mechanisms include the "cross-talk" between Ca^{2+} and cAMP which could be mediated indirectly through Ca^{2+}-dependent protein kinases, such as protein kinase C (Warhurst et al., 1994) or Ca^{2+}/calmodulin dependent protein kinases (Scettini et al., 1984; Hansen et al., 1982; Sharma et al., 1984; Charbonneau et al., 1986; Rossi et al., 1988; Kincaid et al., 1983). In most cases, the precise sites and mechanisms of the synergistic interaction between cAMP and Ca^{2+} effector

pathways have not been clearly characterized.

I.1 Adenylate cyclases and the interaction between calcium and cAMP signalling

The concept of signalling by second messengers originated with the discovery of the role of cAMP (Robison et al., 1968). The cAMP-dependent protein kinase (PKA) has been shown to be important for the hormonal regulation of hormone secretion, such as growth hormone (Narayanan et al., 1989; Spence et al., 1980; Labrie et al., 1983; Sheppard et al., 1979), as well as other cellular events such as glycogenolysis, lipolysis, and catecholamine biosynthesis (Walsh et al., 1994). Studies in adenylate cyclase activity have opened the field of G-protein involvement in cAMP production, as well as many other regulatory processes (Rodbell, 1980, Gilman 1987).

Intracellular Ca^{2+} appears to have at least an equally vital role in cellular events as does cAMP (Berridge, 1993). Intracellular Ca^{2+} plays an important role in neurotransmitter release, such as acetylcholine (del Castillo et al., 1952), hormonal and neurosecretion, such as PTH (Pocotte et al., 1991), GH (Frohman et al., 1986), or insulin (Ashcroft et al., 1994), neuronal excitation, survival of cells, and many other neuronal and non-neuronal functions (Hofmann et al., 1994). As with cAMP, there are Ca^{2+}-initiated cascades (Nishizuka, 1988), such as

Ca^{2+}/calmodulin activated protein kinases; however Ca^{2+} appears to more directly modify other aspects of cellular function, such as GH release (Penner et al., 1988). Studies have shown the importance of G-proteins in adenylate cyclase activity; however current studies suggest other factors, particularly $[Ca^{2+}]i$ may also be important to adenylate cyclase activity (Cooper et al., 1995).

The adenylate cyclase is the prototypical second messenger generator (Cooper et al., 1995). Cellular cAMP levels have been shown to be modulated by the level of $[Ca^{2+}]i$. Such findings suggested that $[Ca^{2+}]i$ and cAMP levels are inextricably interwined. The complexity of this intricate interrelationship between cAMP accumulation and $[Ca^{2+}]i$ was revealed when it was found that there are at least eight adenylate cyclase isoforms, which were multiply regulated (Cooper et al., 1995). It was also found using functional and ultrastructural investigations that adenylate cyclases are intimately associated with sites of Ca^{2+} entry into the cell (Cooper et al., 1995).

I.1.1 Structure of the adenylate cyclase

The cloning of the first adenylate cyclase revealed that the enzyme is a very large, complex structure (Krupinski, 1989). It consists of 1080 - 1248 amino acid residues and are predicted to cross the plasma membrane 12 times, consisting of 2 segments of 6 transmembrane-spanning domains, with each segment followed

by a large cytosolic domain (Feinstein et al., 1991; Bakalyar et al., 1990; Gao et al., 1991; Yoshimura et al., 1992; Premont et al., 1992, Ishikawa, 1992; Katsushika, 1992; Krupinski et al., 1992; Glatt et al., 1993; Wallach et al., 1994; Cali et al., 1994). Although adenylate cyclases resemble ion channels and membrane transporters, there is little or no sequence homology between their transmembrane domains. Also, there is very little sequence homology between each of the eight adenylate cyclases, which may reflect their distinct differences in sensitivity to changing levels of $[Ca^{2+}]i$ (Chen , 1986; Riordan, 1989; Catterall, 1994). Each of the two large cytoplasmic domains contains putative binding sites for ATP. These two binding sites are homologous to one another, and are also homologous between the eight cloned adenylate cyclases (50 - 92%) (Tang et al., 1992; Iyengar, 1993). One proposed functional domain thought to be the site of Ca^{2+}/calmodulin binding to type I adenylate cyclase, a Ca^{2+}-stimulated species, is thought to be in the first cytoplasmic loop, adjacent to the plasma membrane (Vorherr, 1993; Wu et al., 1993).

I.1.2 Multiple regulatory influences of the adenylate cyclases

Of the eight adenylate cyclases that have been cloned and expressed, most, if not all are multiply regulated (Cooper et al., 1995). Conflicting views on universal regulatory mechanisms of adenylate cyclase activity have been dissipated with the discovery of unique multiple influences on each individual adenylate cyclase

isoform. The widely held view that the G_α subunits are the most important regulatory influence on adenylate cyclase activity is being superceded by the fact that protein kinase C, Ca^{2+}, and $\beta\gamma$ subunits of G-proteins can stimulate or inhibit particular adenylate cyclases far more effectively than the G-protein α-subunits (Tang et al., 1992; Iyengar, 1993).

Studies with cells that are transfected with the various adenylate cyclase isoforms have revealed much of this information. For example, phorbol esters (PKC activator) have been shown to increase the stimulation of transfected type II adenylate cyclase twice that of the $G_{s\alpha}$ (Jacobowitz et al., 1993; Lustig et al., 1993). Receptors found to stimulate the $G_{s\alpha}$ subunit were found to minimally stimulate type I or type VIII adenylate cyclases, whereas Ca^{2+} was found to increase the type I and type VIII adenylate cyclases four-fold (Yoshimura et al., 1992; Cali et al., 1994). G_i-linked receptors were only found to inhibit the type VI adenylate cyclase to the same extent that that elevations in $[Ca^{2+}]i$ inhibited the same adenylate cyclase (Boyajian et al., 1991; DeBernardi et al., 1991).

Elevation in $[Ca^{2+}]i$, PKC, the $\beta\gamma$-subunit of the G-protein, and the α-subunit of the G-protein have all been shown to have different effects on the eight different cloned adenylate cyclases. The focus of this study is the regulation of adenylate cyclase activity by elevation in $[Ca^{2+}]i$. An increase in $[Ca^{2+}]i$ was found to stimulate type I, type III, and type VIII adenylate cyclases. Elevated $[Ca^{2+}]i$ was

found to have no effect on the type II, type IV, and type VII adenylate cyclases, while in type V and type VI adenylate cyclases, elevated $[Ca^{2+}]i$ was found to inhibit adenylate cyclase activity. Members of each one of these groupings share regulatory features. However, within these groupings, there is a considerable amount of difference in the range of responses that are generated from increases in $[Ca^{2+}]i$. For example, the concentration of Ca^{2+} that stimulates type I adenylate cyclase is in the normal physiological range (0.1 - 1 μM), whereas type III is stimulated by supraphysiological or supra-normal Ca^{2+} concentrations (> 1000 nM) (Yoshimura et al., 1992; Choi et al., 1992). Of the eight isoforms, no two are regulated in precisely the same manner. As shown in Table 1 (Cooper, 1995, Nature 374:422), there is little redundancy in either the regulation or expression of the multiple isoforms so far detected (Cooper et al., 1995).

The stimulation by Ca^{2+} in types I, III, and VIII adenylate cyclases is mediated through calmodulin, which can be readily removed and added back to restore the Ca^{2+} stimulation of adenylate cyclase activity (Bakalyar et al., 1990; Yoshimura et al., 1992; Tang et al., 1991). It is not sure how Ca^{2+} interacts with types V and VI adenylate cyclases to inhibit activity because there is no recognizable Ca^{2+}-binding sequence, and it does not appear to be working through calmodulin either (Yoshimura et al., 1992; Boyajian et al., 1991).

Some data show that in many cell types there is an organized co-

Table I-1. Properties of cloned mammalian adenylate cyclases grouped by structural relatedness

Adenylate cyclase isoform	Ca^{2+} effect
I	Stimulation
III	Stimulation
VIII	Stimulation
II	No effect
IV	No effect
VII	No effect
V	Inhibition
VI	Inhibition

(Cooper, 1995, Nature 374:422)

localization of adenylate cyclases and Ca^{2+} entry channels, suggesting that the adenylate cyclase sequences include domains that target them to Ca^{2+}-entry sites (Cooper et al., 1995). Where the Ca^{2+} release and entry processes have been separately analyzed, it appears that only Ca^{2+} entering the cell as a result of store depletion, and not Ca^{2+} released from intracellular stores can regulate these adenylate cyclases (Chiono et al., 1995). It remains unclear, in the case of Ca^{2+}/calmodulin-stimulated adenylate cyclases, whether there is a relationship between the apparent concentration of adenylate cyclases and Ca^{2+}-entry sites. Again, see Table 1 (Cooper, 1995, Nature 374:422) for a summary of the regulation of the multiple adenylate cyclase isoforms.

I.2 Phosphodiesterases and the interaction between calcium and cAMP signalling

Early studies on the classification and characterization of phosphodiesterase activity extracted from several tissues soon led to the discovery that multiple phosphodiesterase forms are expressed in mammalian cells (Conti et al., 1991). It is now accepted that there are at least five major families of cyclic nucleotide phosphodiesterases (Table 2; Conti, 1991, Endocr. Rev. 12:220). The Ca^{2+}/calmodulin phosphodiesterases are homodimeric proteins which hydrolyze cGMP with a higher affinity than cAMP, and are activated by Ca^{2+}/calmodulin dependent protein kinase phosphorylation (Hansen et al., 1982; Morril et al., 1979;

Table 2. Phosphodiesterase families of isoenzymes

Family (Abbreviation)	Intracellular modulator	Hormones regulating activity
I. Ca^{2+}/calmodulin PDE's (CaM-PDE)	Ca^{2+}/CaM phosphorylation	Muscarinic cholinergic agonists GnRH
II. cGMP-stimulated PDE's (cGS-PDE)	cGMP	ANF
III. cGMP-inhibited PDE's (cGI-PDE)	cGMP phosphorylation	Insulin Glucagon Dexamethasone
IV. cAMP-specific PDE's (cAMP-PDE)	cAMP	FSH PGE_1 TSH β-Adrenergic agonists
V. cGMP-specific PDE's (cGMP-PDE)	Transducin cGMP???	Light

(Conti, 1991, Endocr. Rev. 12:220)

Sharma et al., 1984; Hansen et al., 1986; Charbonneau et al., 1986).

I.2.1 Structure of phosphodiesterases

There is a consensus that the phosphodiesterases present in mammalian cells have a common structure (Fig. 1; Conti, 1991, Endocr. Rev. 12:221). In the catalytic domain, 270 amino acids are remarkably conserved, suggesting that these residues have an important function (Charbonneau, 1990; Le Trong et al., 1990; Sass et al., 1986; Conti et al., 1990). Proteolysis of a Ca^{2+}/calmodulin dependent phosphodiesterase purified from bovine brain results in a proteolytic fragment that can still hydrolyze nucleotides, but cannot be regulated by a Ca^{2+}/calmodulin complex (Charbonneau, 1990; Tucker et al., 1981; Kincaid et al., 1985), indicating that the catalytic domain can be separated from the regulatory domains.

I.2.2 Phosphodiesterase activation by intracellular Ca^{2+}

In membrane preparations, Ca^{2+}/calmodulin phosphodiesterase forms, which have been isolated and characterized (Sharma et al., 1980; Kincaid et al., 1983), have been shown to be activated by Ca^{2+}/calmodulin complexes, thereby decreasing cAMP levels. Changes in $[Ca^{2+}]i$ levels produce changes in Ca^{2+}/calmodulin dependent phosphodiesterase activity, and consequently

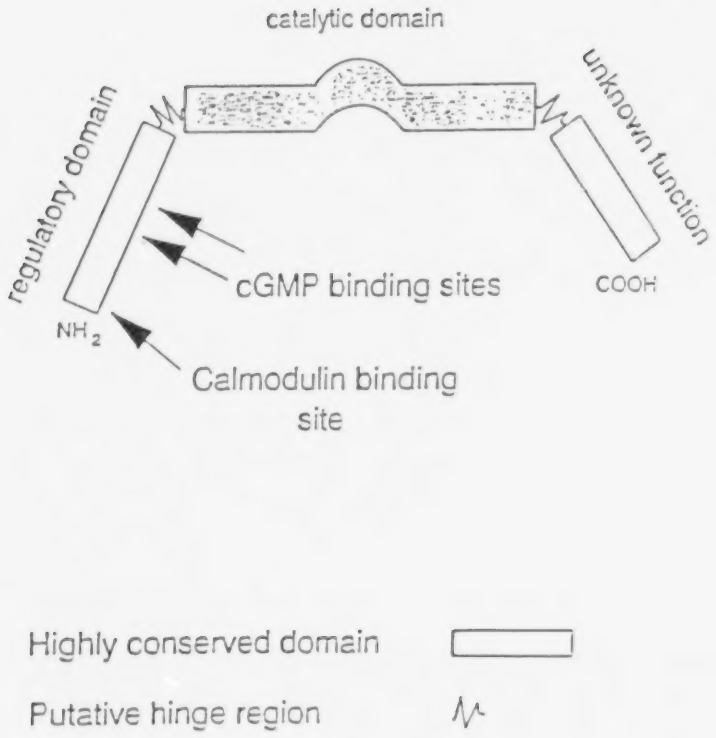

Figure I-1. Structure of the cyclic nucleotide phosphodiesterases (Conti, 1991, Endocr. Rev. 12:221)

fluctuations in intracellular cAMP accumulation (Fig. 2; Conti, 1991, Endocr. Rev. 12:227). In several cell types, such as $1321N_1$ human astrocytoma cell lines or dog thyroid slices, intracellular cAMP levels were shown to be inhibited by Ca^{2+}-mobilizers, such as muscarinic cholinergic agonists (Harden et al., 1985; Meeker et al., 1982; Meeker et al., 1983) and carbachol (Erneaux et al., 1985; Van Sande et al., 1977; Van Sande et al., 1979), respectively. These two Ca^{2+}-mobilizers involve phosphatidyl inositol turnover, which causes an increase in $[Ca^{2+}]i$, and activation of a Ca^{2+}/calmodulin dependent phosphodiesterase. A similar increase in the rate of cAMP degradation was obtained using the Ca^{2+}-ionophore, A23187, in astrocytoma cells, indicating that increases in $[Ca^{2+}]i$ do play a role in the activation of Ca^{2+}-calmodulin dependent phosphodiesterases, when conducted in certain cell types (Meeker et al., 1982; Van Sande et al., 1979; Miot et al., 1984; Tanner et al., 1986). Again, Table 2 (Conti, 1991, Endocr. Rev., 12:220) summarizes the regulation of the various forms of phosphodiesterases.

I.3 Interaction between Ca^{2+} and GHRH-stimulated cAMP accumulation

Cyclic AMP and $[Ca^{2+}]i$ have been shown to be interrelated in the regulation of secretory processes for a variety of hormones, such as prolactin (Schettini et al., 1983), insulin (Valverde et al., 1979), and MSH (Tsuzata et al., 1982). Many of the regulatory functions of Ca^{2+} are mediated through a Ca^{2+}/calmodulin dependent kinase (Rasmussen , 1981; Cheung, 1980; Means et al., 1980). For

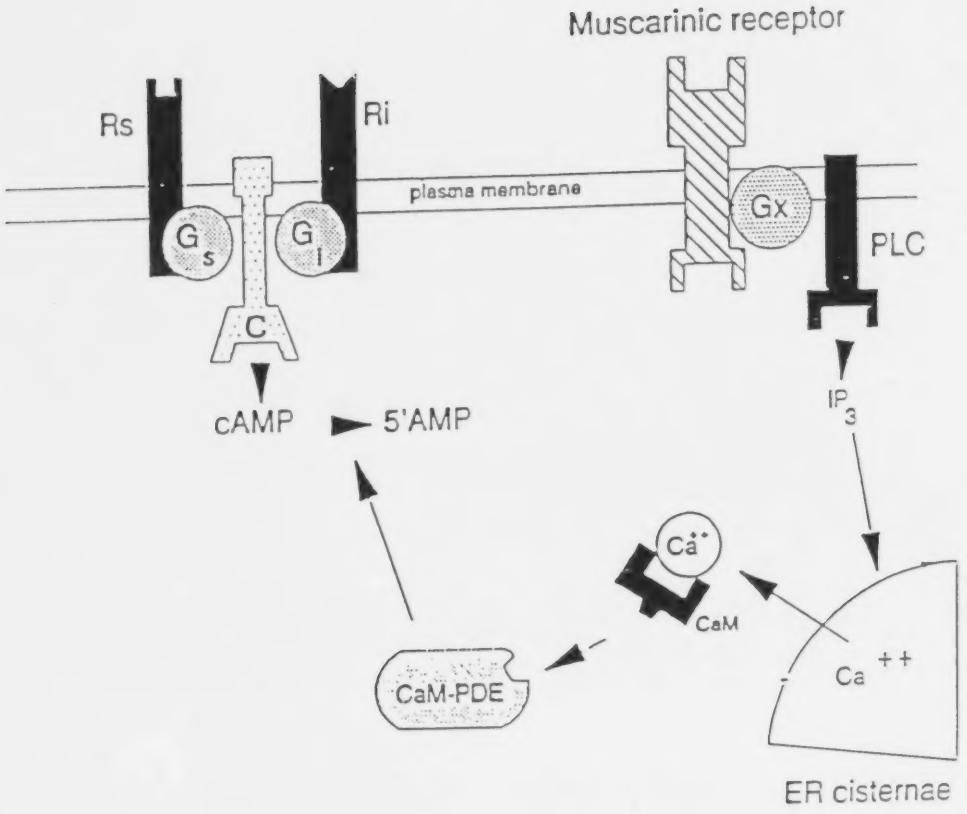

Figure I-2. Mechanism of regulation of the Ca^{2+}/calmodulin dependent phosphodiesterases by those hormones that regulate phosphatidyl inositol turnover and intracellular calcium

(Conti, 1991, Endocr. Rev. 12:221)

example, Ca^{2+}/calmodulin dependent protein kinase mediates the stimulation by Ca^{2+} of the adenylate cyclase in brain (Brostrom et al., 1975; Cheung et al., 1975), adrenal medulla (LeDonne et al., 1979), pancreatic islet (Valverde et al., 1979), and the pituitary gland (Schettini et al., 1984).

In the case of anterior pituitary cells, the effect of $[Ca^{2+}]i$ on the growth hormone-releasing hormone (GHRH)-stimulated cAMP accumulation appears to be very controversial (Frohman et al., 1986). However, it seems certain that Ca^{2+} is necessary for the GHRH-stimulated cAMP accumulation (Frohman et al., 1986). While it has been shown that the Ca^{2+}-ionophore, A23187, enhanced the GHRH-stimulated cAMP response (Schettini et al., 1984), low Ca^{2+} medium also resulted in a similar effect (Lussier et al., 1987). Also, Ca^{2+} has been reported to produce a concentration-dependent inhibition of basal- and GHRH-stimulated adenylate cyclase activity (Narayanan et al., 1989). This suggests that the regulation of the GHRH-stimulated cAMP accumulation by $[Ca^{2+}]i$ is very complex. Different $[Ca^{2+}]i$ elevating agents may have distinct effects on the cAMP system depending on the mechanism through which $[Ca^{2+}]i$ is elevated by these agents. Furthermore, since the A23187-induced elevation of $[Ca^{2-}]i$ is commonly above the range of physiological relevance (Warhurst et al., 1994), other Ca^{2+}-dependent enzymes, which normally do not participate in the regulation of cAMP, may be activated.

I.4 Thapsigargin and the activation of Ca^{2+} entry

Tg is a sesquiterpene lactone tumour promoter isolated from the umbelliferous plant Thapsia garganica L. (Apiaceae) (Rasmussen et al., 1978). Tg has been shown to induce Ca^{2+}-dependent responses in a variety of cell types (Takemura et al., 1990). Also, Tg elevates $[Ca^{2+}]i$ by a mechanism that involves mobilization of intracellular Ca^{2+} stores (Thastrup et al., 1987; Patkar et al., 1979). Tg was found to act in a different manner than Ca^{2+}-ionophores, such as A23187 (Thastrup et al., 1989). Thapsigargin mobilizes a pool of Ca^{2+} that was shared by bradykinin. However, unlike bradykinin, Tg did not stimulate inositol phosphate formation (Jackson et al., 1988). More recently, Tg has been shown to specifically release Ca^{2+} from microsomes by the inhibition of the Ca^{2+}-ATPase and active transport responsible for the accumulation of Ca^{2+} into a non-mitochondrial (1,4,5) IP_3-sensitive intracellular pools (Thastrup et al., 1989).

I.4.1 History of thapsigargin

Tg and thapsigarcin are two major active components (resins) that are found in the root extracts of the umbelliferous plant, Thapsia garganica (Rasmussen et al., 1978). This root extract had been used as a counter-irritant for the relief of rheumatic pains, joint inflammation, and treatment of sore muscles. Chemical investigations, using NMR, circular dichroism spectroscopy, and

crystallographic methods, have revealed that the major active constituent of Thapsia garganica is a sesquiterpene lactone of the guaianolide type (Christensen, 1983; Christensen, 1985). This alcohol extract of the root Thapsia garganica had a molecular weight of 650, and mainly consisted of carbon, oxygen, and hydrogen, but not nitrogen (Patkar et al., 1979). The compound that was extracted was named thapsigargin (Fig. 3; Sabala, 1993, Acta Bioch. Pol. 40:310). Tg has been shown to elicit Ca^{2+}-dependent responses in a variety of cell types, such as platelets (platelet activation) (Thastrup et al., 1987), mast cells (histamine release) (Patkar et al., 1979; Jacobsen et al., 1987; Ali et al., 1985), neutrophils (Kano et al., 1987), and NG115-401L neuronal cells (Jackson et al., 1988).

I.4.2 Mechanisms of action of thapsigargin

Tg, as previously mentioned, has been shown to induce elevated $[Ca^{2+}]i$ in cells by a mechanism involving emptying of intracellular stores. Tg, also as stated before, is not a structurally distinct Ca^{2+} ionophore. This possibility was ruled out by several lines of evidence.

(1) Tg cannot transfer Ca^{2+} from an aqueous phase into an organic phase, thus allowing Ca^{2+} to pass through the plasma membrane.

(2) Tg is not able to induce Ca^{2+} release of K^+ from erythrocytes

(3) Tg does not saturate the trapped intracellular divalent cation-

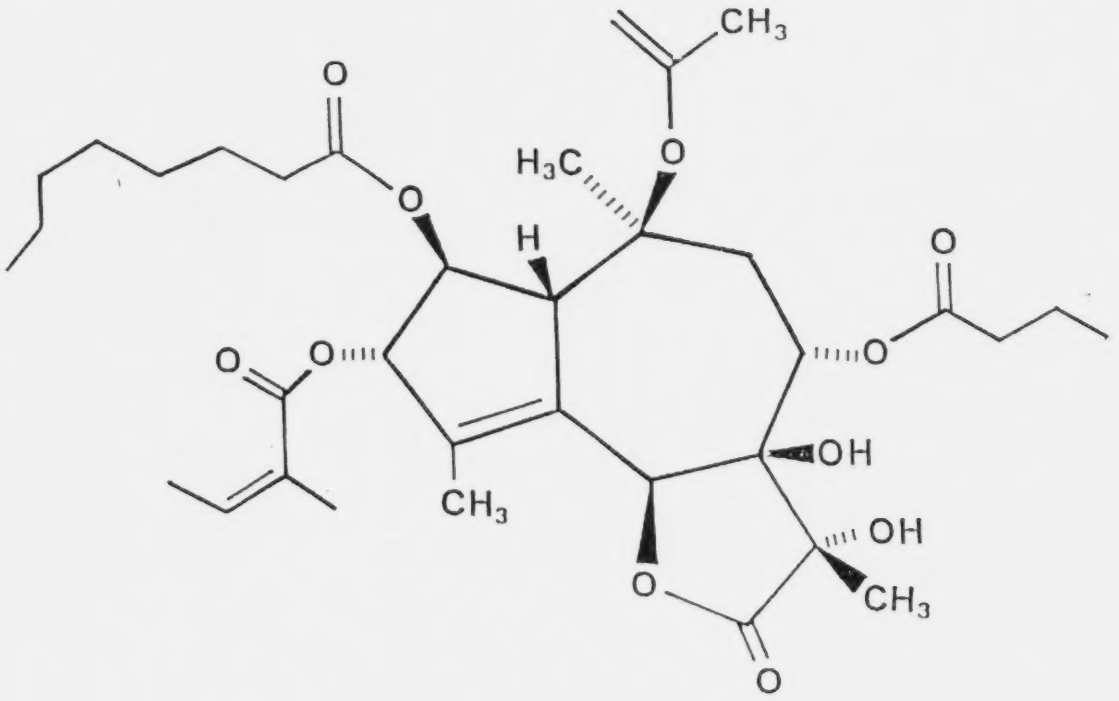

Figure I-3. Structure of thapsigargin (Sabala, 1993, ACTA Bioch. Pol. 40:310)

sensitive probes, Quin-2 or Fura-2 (Jackson et al., 1988; Thastrup et al., 1989)

Tg is able to induce tumours (second stage carcinogenesis in mice) through the elevation of $[Ca^{2+}]i$ in a manner that is independent of activation of protein kinase C or a protein phosphatase (Jackson et al., 1988; Fujiki et al., 1986). Therefore, Tg is called a non-PMA type tumour promoter because PMA activates protein kinase C, which in turn promotes tumours (Jackson et al., 1988; Fujiki et al., 1986). We will now look at the specific mechanisms of action of Tg in several cell types.

I.4.3 Thapsigargin's effect on cytoplasmic Ca^{2+} concentrations

In platelets, hepatocytes, and neuronal cells, Tg produced a pronounced increase in $[Ca^{2+}]i$ (Thastrup et al., 1989). However, each of these types of cells reacted differently to Tg. In platelets and hepatocytes, there was a $[Ca^{2+}]i$ increase, which was, in turn maintained at this new elevated state. In the neuronal cells, there was a transient $[Ca^{2+}]i$-rise, which returns to basal level. The sustained plateau of $[Ca^{2+}]i$, as observed in platelets and hepatocytes, is most likely due to a change in steady state levels of Ca^{2+} by either Ca^{2+}-entry from the extracellular space into the cytoplasm, or Ca^{2+} from the intracellular stores (Thastrup et al., 1989). The removal of extracellular Ca^{2+}, by the Ca^{2+}-chelator, EGTA, showed that

the initial, pronounced part of the Tg-induced $[Ca^{2+}]i$ rise is obtained by the discharge from internal Ca^{2+} stores (Thastrup et al., 1989). Additional stimulated entry, or the new sustained levels of $[Ca^{2+}]i$, by Tg was substantiated by the ability of Tg to induce the influx of extracellular Ca^{2+}. In this particular study, Mn^{2+} was used as a marker because Mn^{2+}, it was believed, competes with Ca^{2+} in passing through agonist opened Ca^{2+} channels (Merritt et al., 1988), and then may be readily detected over Ca^{2+} because Mn^{2+} binds with higher affinity to the indicator dye, Fura-2 AM. Therefore, Merritt et al. (1988) suggested that the extracellular influx component of the Tg-induced $[Ca^{2+}]i$-rise is secondary or delayed in comparison to the initial, transient intracellular Ca^{2+} mobilizing component (Fig. 4; Takemura, 1990, J. Biol. Chem. 264:12270).

The absence of this secondary or extracellular influx of Ca^{2+} is seen in neuronal cells (Jackson et al., 1988; Hanley et al. 1988). This is indicated by the return of the $[Ca^{2+}]i$ signal to baseline level. This implies that in the neuronal cells Ca^{2+}-influx from the extracellular space is absent. This effect of Tg may have important implications, in that different cells may have different mechanisms for the induction of Ca^{2+}-entry. Thus, Tg may be used as a tool to better understand refilling of the depleted intracellular Ca^{2+} stores, without the concomitant formation of inositol phosphates (Thastrup et al. 1989).

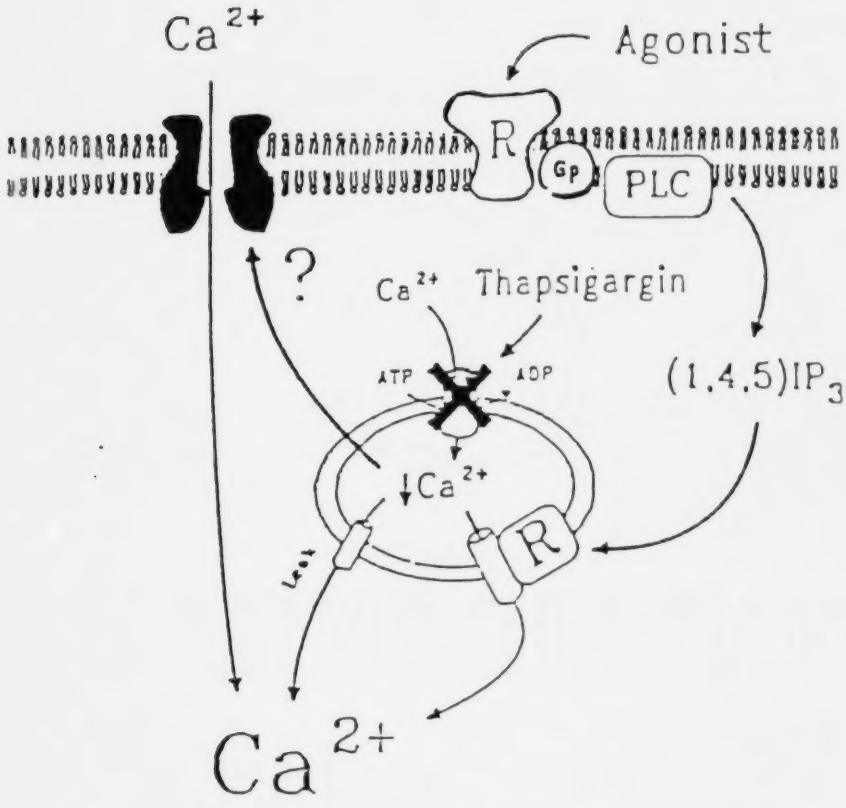

Figure I-4. Model for the regulation of Ca^{2+}-fluxes by 1,4,5-IP3 and thapsigargin (Takemura, 1990, J. Biol. Chem. 264:12270)

I.4.4 Thapsigargin's effect on the hydrolysis of phospho-inositides

To answer the question of whether breakdown of phospho-inositides and the resulting production of inositol phosphates were involved in the Ca^{2+}-mobilizing activity of Tg, Tg was examined first by its ability to induce phosphorylation of platelet proteins. This study by Thastrup et al. (1989) compared the effects of thrombin and Tg on the phosphorylation of these platelet proteins. They found that Tg's phosphorylation was less intense than thrombin, and also developed more slowly. Since thrombin was well documented in its activation of PKC through PIP_2 breakdown into diacylglycerol and 1,4,5-IP3 production, it was thought that perhaps Tg activated PKC, as well as Ca^{2+}-mobilization in its platelet activating effect. However, pretreatment of the platelets with acetylsalicylic acid strongly inhibited the Tg-induced phosphorylation of the characteristic 47 kDa protein substrate for PKC, without significant effect on the 20 kDa protein substrate for the Ca^{2+}-calmodulin kinase. These findings by Thastrup et al. (1989) suggest that Tg may be selectively targeted to mobilization of intracellular Ca^{2+} alone, and not the hydrolysis of PIP_2, which is needed for protein kinase C production. The inhibitory action of aspirin (phospholipase A2 inhibitor), on the other hand, suggests that the phospholipase A2 enzyme may be activated as a result of $[Ca^{2+}]i$ elevations alone, which in turn activates PKC.

Studies with cloned mammalian neural cell line NG115-401L indicated that

although Tg provoked an intracellular Ca^{2+}-discharge, it did not provoke a concomitant production of any inositol phosphates (Jackson et al., 1988). Jackson et al. (1988) showed clearly that there was no production of ^{3}H-inositol phosphate by Tg when compared to the 1,4,5-IP3 production by bradykinin. Thus, it appears Tg does not act via the phospholipase C pathway. Also, Tg does not appear to activate any plasma membrane receptor, G_o protein, or catalytic unit, such as phospholipase C or adenylate cyclase (Thastrup et al., 1989).

I.4.5 Thapsigargin's discharge of intracellularly sequestered Ca^{2+}

The ability of Tg to evoke release of Ca^{2+} from an intracellular pool, suggests that it may share a common site of action with 1,4,5-IP3. One such site could be an associated endoplasmic reticulum (ER) channel (Thastrup et al., 1989). Thastrup et al. (1989) added Tg to Ca^{2+}-loaded microsomal membrane vesicles, which led to a rapid and complete release of the sequestered Ca^{2+}. Tg was seen to release essentially all Ca^{2+}, whose uptake was ATP-dependent (Thastrup, 1990). Overwhelming evidence that Tg emptied Ca^{2+} from overlapping microsomal Ca^{2+} pools was seen where the Ca^{2+}-releasing ability of GTP and 1,4,5-IP3 are abolished by Tg pretreatment. The amount of Tg-releasable Ca^{2+} was also decreased by pretreatment with either GTP or 1,4,5-IP3. Tg and 1,4,5-IP3, in contrast to GTP, released Ca^{2+} immediately from Tg-sensitive pools. However, the initial rate of Ca^{2+}-release was found to be much faster for 1,4,5-IP3

than Tg (Thastrup et al., 1989). Evidence for a common intracellular Ca^{2+} store is also seen in the study by Takemura et al. (1990). In this study, rat acinar cells, in the absence of extracellular Ca^{2+} were treated with methacholine, an agonist which mobilizes Ca^{2+} through 1,4,5-IP3 production. This inhibited the response of the cells to Tg. Likewise, pretreatment of the cells with Tg inhibited the response of the cells to methacholine. The simultaneous addition of Tg and methacholine produced a greater initial release of Ca^{2+} than either themselves, but they were not additive in the sustained phase of Ca^{2+} mobilization. These results demonstrate, again, that Tg and methacholine share the same intracellular store for the initial release of Ca^{2+}. Also shown by this study was that both Tg and methacholine induced the influx of Ca^{2+} from the extracellular space (Takemura et al., 1990). Tg shows that it is the depletion of the intracellular pool that mediates the Ca^{2+}-mediated Ca^{2+} influx. This is consistent with the "capacitative model", which states that the entry of Ca^{2+} from the extracellular space is initiated by the depletion of the intracellular inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pools (Putney, 1986).

I.4.6 The inhibitory effect of thapsigargin on Ca^{2+}-ATPase activity

The ATP-driven accumulation of Ca^{2+} into microsomes was completely blocked by Tg pretreatment, suggesting that the effects of Tg were to inhibit uptake of Ca^{2+} into 1,4,5-IP3-sensitive pools, rather than a direct stimulation of an

efflux process, like 1,4,5-IP3. Support for this possibility was provided by Thastrup et al. (1989) using a hexokinase/glucose treatment, which uses up all the ATP present, which, in turn, also induced Ca^{2+} release, with kinetics and maximum effect very similar to those produced by Tg, suggesting that Tg is acting on a process which is ATP dependent (Fig. 5; Thastrup, 1990, Proc. Natl. Acad. Sci. USA 87:2468). Direct measurement of Ca^{2+}-ATPase activity also revealed that Tg dose-dependently inhibited the Ca^{2+}-ATPase activity directly (Fig. 6; Thastrup, 1990, Proc. Natl. Acad. Sci. USA 87:2468) (Thastrup et al., 1990).

This inhibitory effect of Tg on the 1,4,5-IP3 sensitive ER Ca^{2+}-ATPase is highly specific, as basal Mg^{2+}-ATPase in this membrane fraction was unaffected. Also, Tg produced no inhibitory effect on Ca^{2+} efflux from loaded plasma membrane vesicles. Tg, therefore, must interact with a specific domain on the ER Ca^{2+}-ATPase (Thastrup et al. 1990).

Furthermore, this led into another study, in which Tg potently inhibited the ER Ca^{2+}-ATPase, but it was a much less potent inhibitor of cardiac sarcoplasmic reticulum Ca^{2+}-ATPases, and is apparently ineffective against the enzymes from skeletal muscle sarcoplasmic reticulum and plasma membrane Ca^{2+}-ATPases (Thastrup et al., 1990). This suggests Ca^{2+}-ATPases of the ER differ significantly from other isoforms. It has been shown that the ER Ca^{2+}-ATPase is

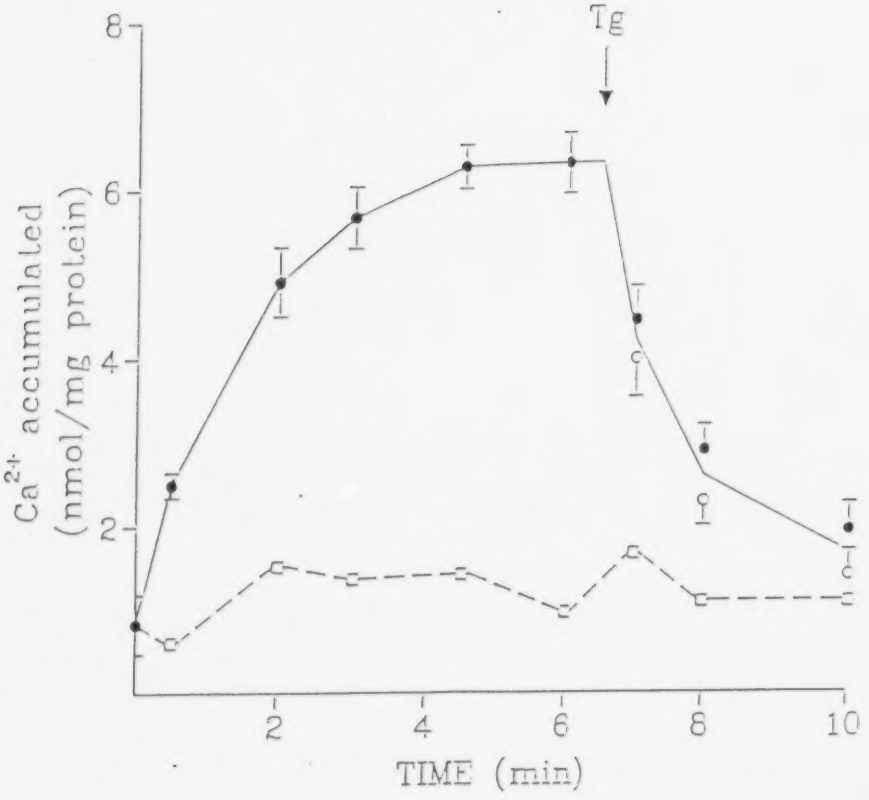

Figure I-5. Effect of thapsigargin on uptake and efflux of $^{45}Ca^{2+}$ by rat liver microsomes. ●, Thapsigargin (Tg, 68 nM) was added at t = 6.5 min; ○, hexokinase/glucose (11.2 units per ml/4.5 mM) mixture was added at t = 6.5 min; □, thapsigargin (68 nM) was added at t = 0 min. Experiments were performed at 37°C. Data points represent the mean of three experiments, and the bars show SD.

(Thastrup, 1990, Proc. Natl. Acad. Sci. USA 87:2468)

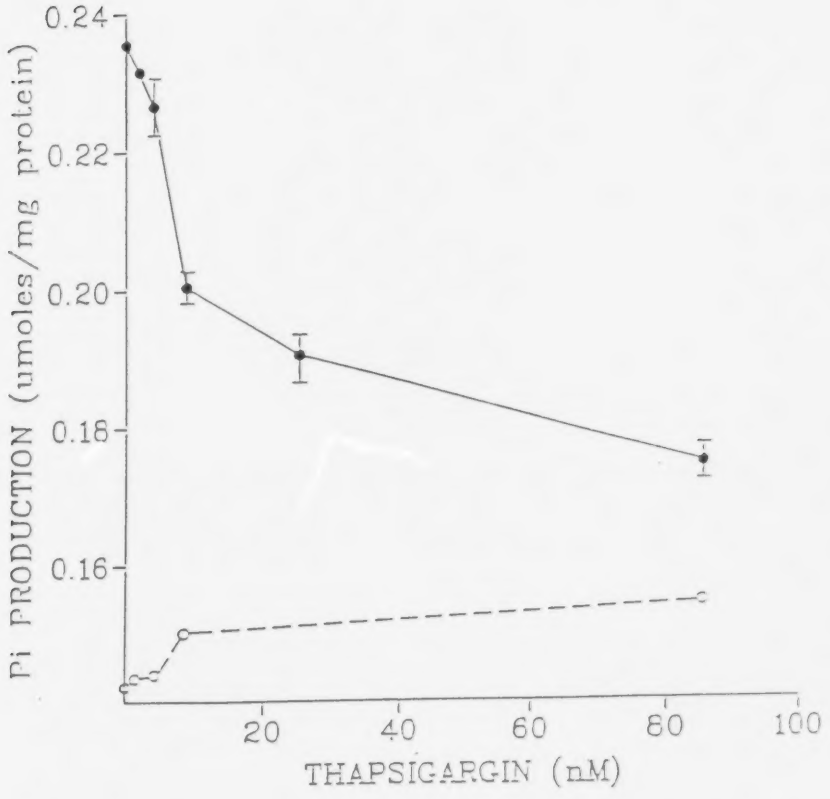

Figure I-6. Inhibition of the microsomal Ca^{2+}-ATPase by thapsigargin in the presence (●) or absence (○) of Ca^{2+}. Experiments were performed at 37°C. Data are the means ± SD of four experiments.

(Thastrup, 1990, Proc. Natl. Acad. Sci. USA 87:2468)

immunologically similar to the cardiac enzyme and differs significantly from skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPases and plasma membrane Ca^{2+}-ATPases (Lytton, 1988). Cloning of the multiple molecular forms of Ca^{2+}-ATPases has revealed that the predominant form likely corresponding to the ER Ca^{2+}-ATPase (Lytton, 1988), has an extended C-terminal tail with another transmembrane region. This extended C-terminus of the ER Ca^{2+}-ATPase may provide some answers as to the specificity of the interaction of Tg and ER Ca^{2+}-ATPases, rather than the plasma Ca^{2+}-ATPases, cardiac sarcoplasmic reticulum Ca^{2+}-ATPases, or the skeletal muscle Ca^{2+}-ATPases. At present it is not known whether Tg induces the ER Ca^{2+}-ATPase itself to act as a direct pathway for Ca^{2+} release, or whether there is a permanent Ca^{2+} leakage pathway present, likely situated at a separate molecular site in the ER.

I.4.7 Thapsigargin as a pharmacological tool

Tg provides us with a pharmacological tool that is able to specifically modulate the levels of $[Ca^{2+}]i$, without interrupting other signal transduction pathways inside the cell (ie. generation of inositol phosphates, protein kinase C, or diacylglycerol). There are many different examples of how Tg is used as an ER Ca^{2+}-ATPase inhibitor, to elicit elevated levels of $[Ca^{2+}]i$ within a physiologically relevant range, unlike many Ca^{2+} ionophores, and create a physiological response (Warhurst et al., 1994).

I.5 Thapsigargin and the cAMP signal transduction pathway

Other studies have shown that the administration of Tg to C6 glioma cells simultaneously with isoproterenol, which stimulates cAMP accumulation, inhibited the isoproterenol-stimulated cAMP synthesis by 68% (Lin et al., 1993). This study by Lin et al. (1993) suggested that it is possible that Tg's Ca^{2+}-depleting activity of the ER was somehow responsible for the inhibition of the adenylate cyclase activity. Also suggested by this study was that by administering 3-isobutyl-1-methylxanthine (IBMX), the phosphodiesterase inhibitor, that the inhibitory effects of Tg on cAMP production could be partially due to a Ca^{2+}-dependent stimulation of phosphodiesterase activity.

I.6 Specific aim of the present study

Several intracellular pathways have been shown to be involved in the release of GH. Increased cAMP accumulation, via a cAMP dependent protein kinase, converts non-functional Ca^{2+} channels to a functional form, which leads to a large, rapid increase in $[Ca^{2+}]i$, thus promoting the release of GH via the process of exocytosis (Lussier et al., 1991). Studies with anterior pituitary cells have demonstrated that dibutyryl cAMP-mediated GH release was biphasic: an initial rapid rate of hormone release at 15 to 20 minutes which was followed by a second release beginning at 60 min (Stachura, 1976). Such phasic release was consistent

with similar observations by other investigators with other secretagogues and other hormones held in granular storage (insulin, glucagon, vasopressin, and luteinizing hormone), and suggested the availabilty for release of two pools of stored hormone (Stachura, 1982). The first or labile pool was available for immediate release, while the second pool was available for prolonged release at an increased rate under constant stimulation. It has also been found that, in addition to $[Ca^{2+}]i$ having a direct role in the release of growth hormone (Frohman et al., 1986), $[Ca^{2+}]i$ has been shown to interact directly with the synthesis of cAMP (Brazeau et al., 1982).

With these findings in mind, it was decided to study the effects of $[Ca^{2+}]i$, using different $[Ca^{2+}]i$ mobilizing agents, on the regulation of the GHRH-stimulated cAMP pathway in rat anterior pituitary cells. Rat anterior pituitary cells were chosen because both $[Ca^{2+}]i$ and cAMP have been found to play important roles in the release of GH (Frohman et al., 1986). Tg was chosen as the $[Ca^{2+}]i$ elevating agent because, unlike other $[Ca^{2+}]i$ elevating agents, like Ca^{2+}-ionophores, Tg elevates $[Ca^{2+}]i$ within a physiologically relevant range. Also, using Tg as a pharmacological tool allows minimal interference with other physiological pathways, such as inositol phosphates formation or PKC activation.

Therefore, the purpose of this thesis is to examine the effect of elevation of $[Ca^{2+}]i$ on the GHRH-stimulated cAMP accumulation using the Ca^{2+}-mobilizer, Tg.

Specifically, this study examined: (1) the effect of Tg on $[Ca^{2+}]i$ in anterior pituitary cells; (2) the effect of Tg on GHRH-, PACAP-, cholera toxin-, and forskolin-stimulated cAMP accumulation; (3) the sites of action of Tg on the GHRH-stimulated cAMP accumulation; (4) the mechanisms through which Tg modulated the GHRH-stimulated cAMP accumulation, in particular, whether protein kinase(s) were involved in the effect of Tg on GHRH-stimulated cAMP accumulation; (5) the effect of Tg versus another Ca^{2+} elevating agent, K^+, on $[Ca^{2+}]i$ and GHRH-stimulated cAMP accumulation; and (6) the effect of Tg and K^+ on GH release.

II. Materials and Methods

II.1 Materials

Synthetic rat GHRH and pituitary adenylate cyclase-activating polypeptide 1-38 (PACAP) were obtained from Peninsula Laboratories (San Carlos, CA). Synthetic rat GHRH was initially dissolved and stored in sterile double-distilled water at a concentration of 10^{-5} M. Further dilutions were made in sterile double-distilled water. Trypsin, DNAase, albumin, and trypsin inhibitor for the cell preparation were obtained from Sigma Chemical Corp. (St. Louis, MO). The acetoxy-methyl ester of fura-2 (fura-2 AM) and BAPTA (BAPTA-AM), which are both cell permeable, were purchased from Molecular Probes Inc. (Eugene, OR). Dulbecco's modified eagle medium (DMEM) were purchased from Gibco (Grand Island, NY). Cholera toxin, isobutylmethylxanthine (IBMX), (1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), culture medium, and fetal bovine serum, Triton X-100, EGTA, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Corp. (St.Louis, MO). Forskolin and calphostin C were obtained from Calbiochem (La Jolla, CA). Tg was obtained from Research Biochemicals International (Natick,MA). [^{125}I]cAMP was obtained from ICN Immunobiologicals (Lisle, IL). ^{125}I-labelled NaI for iodination of GH was obtained from Amersham (Oakville, Ontario). All other drugs and chemicals were obtained from commercial sources and were

of the purest grade available. Antibodies for the RIA of cAMP were gifts from Dr. A. Baukal (National Institute of Health, Bethesda, MD). The antigen, antiserum, and the reference preparation for the RIA of GH were obtained from the National Institute for Arthritis, Diabetes, Digestive and Kidney Disease (NIADDK, Baltimore, Maryland). Sheep anti-monkey antiserum and normal monkey serum were kind gifts of Dr. G.M. Brown (Clark's Institute of Psychiatry, Toronto, Ontario).

II.2 Cell Culture

a) Enzymatic dissocation by trypsinization

Male Sprague-Dawley rats (180-200g) were decapitated, the pars nervosa-intermedia were discarded, and the anterior pituitary glands were collected in ice-cold phosphate-buffered saline (Appendix 1). The glands were washed 3 times with phosphate-buffered saline (PBS) and then minced into small fragments. The fragments were then transferred to the digestion medium consisting of Dulbecco's Modified Eagle Medium (DMEM) with trypsin (1 mg/ml) and DNAase (0.01%,vol/vol). The glands were allowed to incubate in the medium at 37°C in a shaking water bath. After 12-15 min, the reaction was stopped with fetal calf serum (10%, vol/vol). The entire solution was then centrifuged at 1500 x g for 8 min. The supernatant was discarded and the pellet was resuspended in PBS. Dispersion was carried out by triturating the glands with a pipette. The chunks

were allowed to settle down and the supernatant was transferred to a centrifuge tube. The trituration was repeated until all the tissue was dispersed. The cell suspension was then centrifuged at 1500 x g for 8 min. The supernatant was discarded and the pellet was resuspended in culture medium (DMEM with 10% Fetal Calf Serum [FCS]). The cells were washed twice and resuspended in the culture medium. Cell count and viability were determined using trypan blue dye exclusion method. Cell count was about 10^6/gland. Viability was about 90%. The cells were then plated onto multi-welled dishes at a density of 1.25-1.50 X 10^5 cells/well in 0.3 ml of culture medium. The cells were then incubated under a humidified atmosphere of 95% air/ 5% CO_2 at 37°C. After 48 h, the cells were washed three times with DMEM (without FCS) and equilibrated for 30 min before performing the experiments.

II.3 Drug treatment

The pituitary cells plated at a concentration of 1.25-1.50 x 10^5 cells per well were treated with various drugs. Drugs were dissolved in 200X concentrated solution in water or DMSO before diluting to the final concentration in DMEM with 0.1% bovine serum albumin (pH 7.4). The plated cells were washed a third time, and then the medium bathing the cells was replaced medium in which the drugs were dissolved to the required concentration. The treatment period was 15 min unless indicated otherwise. At the end of the treatment period, the medium was

removed and assayed for GH release. Acetic acid (5mM) was then added to the plate and frozen on dry ice immediately. The plated cells were lysed by alternate freezing and thawing in 5 mM acetic acid and intracellular accumulation of cAMP was measured.

II.4 Radioimmunoassay for cAMP

For cAMP measurements, the medium was boiled for 5 min and assayed using a radioimmunoassay procedure in which samples are acetylated prior to analysis (Harper et al., 1975; Ho et al., 1989). For the RIA of cAMP, a 2-day double antibody radioimmunoassay protocol was used. On the first day, the standards and the samples were added to glass test tubes followed by the sodium acetate (NaAc) buffer. The radiolabelled ligand was added to all the tubes, while the first antibody (B4) was added to all the tubes except for the total and the non-specific binding tubes. The tubes were then allowed to incubate at -4^{0}C for 24h. On the second day, the second antibody (sheep anti-rabbit), the carrier (normal rabbit serum), and polyethylene glycol were added. After incubation for 1h, the tubes were centrifuged at 3200 x g for 45 min at 4°C. The supernatant was discarded and the precipitate (pellet) was assessed using a gamma counter (Cobra Auto-gamm, Canberra Packard, Mississauga, Ontario). Intra- and interassay coefficients of variation were < 10%. All data are presented as mean +/- standard error of the mean (SEM) of cAMP concentration levels in four

aliquots of wells. The results are expressed as picomoles/300,000 cells.

II.5 Radioimmunoassay for Growth Hormone

Growth hormone was assayed using the National Institute for Arthritis, Diabetes, Digestive, and Kidney disease rat radioimmunoassay kits. Growth hormone was assayed in quadruplicate by the double antibody RIA and the results are expressed as nanograms / 300,000 cells. For the RIA of GH, a 3-day double antibody radioimmunoassay protocol was used. On the first day, the standards and the samples were added to glass test tubes followed by the GH buffer (1% BSA in 0.025 M EDTA phosphate-buffered saline, pH 7.6, Appendix 2). The antibody (monkey anti-rGH) was then added and allowed to incubate at -4°C for 24 h. On the second day, the radiolabelled ligand was added to all tubes, which were then allowed to incubate at room temperature overnight. On the third day, the second antibody (sheep anti-monkey gamma globulin) and the carrier (normal monkey serum) were added and allowed to incubate at room temperature for 3 h. Polyethylene glycol was then added, and the remaining steps were identical to those used in the cAMP radioimmunoassay procedure.

II.6 Determination of intracellular Ca^{2+}

Intracellular Ca^{2+} was determined using a fluorescent Ca^{2+} indicator fura-2

(Rink et al., 1982; Ho et al., 1991). Cells (5×10^5) were pelleted and resuspended in culture medium (DMEM with FCS, pH 7.2). The cells were loaded by incubation with 5μM fura-2 AM for 45 min at 37°C. The cells were then pelleted, washed twice and resuspended in a fresh buffered salt solution which contained (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1.2, KH_2PO_4 1.2, HEPES 25, and glucose 6: pH 7.2. Aliquots of this suspension (1.5 ml) were transferred to a cuvette for the fluorometric determination of $[Ca^{2+}]i$, using a SLM Aminco DMX1000 fluorescence spectrophotometer with a thermostatically controlled cell holder fitted with a magnetic stirrer. Intracellular Ca^{2+} of pituitary cells was determined by monitoring the ratio of the fluorescence emission signal at 510nm, with the excitation wavelengths set at 380 nm and 340 nm. The temperature was maintained at 37°C. Fluorescence signals were recorded continually with a computer. Free Ca^{2+} concentration was calculated according to the equation established by Poenie et al. (Poenie et al., 1985): $[Ca^{2+}]i = K_d \times F_0/F_s \times (R-R_o)/(R_s-R)$ where K_d is the dissociation constant of fura-2-Ca^{2+} complex (225nM), F_o and F_s are the fluorescence intensities at 380 nm for free (o) and Ca^{2+}-saturated (s) dye, and R, R_o, and R_s are the ratio of the dye fluorescence intensities at 340 nm and 380 nm for unknown, free and Ca^{2+}-saturated dye respectively. Both F_s and R_s were determined by lysing the cells with Triton X-100 (0.1%) while F_o and R_o were determined by addition of 10mM EGTA to the lysed cell suspension.

II.7 Statistical analysis

Data are presented as the means +/- SEM of the amount of cAMP and GH in four aliquots of cells. Each experiment was repeated at least three times from different cell preparations which generated consistent results. Statistical comparisons were analyzed by unpaired-*t* tests for cAMP determination and paired-*t* tests for $[Ca^{2+}]i$ measurements. Statistical significance was set at $P < 0.05$.

III. Results

III.1. Effect of Tg and GHRH on $[Ca^{2+}]i$

a) Effect of Tg on $[Ca^{2+}]i$

Tg has been shown to elevate $[Ca^{2+}]i$ in a variety of cell types. In this study, the effect of Tg on $[Ca^{2+}]i$ in rat anterior pituitary cells was examined using fura-2 AM. The resting $[Ca^{2+}]i$ of anterior pituitary cells was 189 +/- 12.9 nM (n = 4). Addition of Tg (20 μM) caused a large transient increase in $[Ca^{2+}]i$ followed by a small and sustained increase which lasted at least 10 min (Fig. 7). The peak $[Ca^{2+}]i$ was 460 +/- 15.9 nM and the sustained component was 263 +/- 14.3 nM. The Tg mediated increase in $[Ca^{2+}]i$ was concentration-dependent (between 0.2 μM to 20 μM). The initial peak of $[Ca^{2+}]i$ for Tg (20 μM) occurred immediately after addition, while the initial peak for Tg (2 μM and 0.2 μM) was not apparent.

b) Effect of GHRH on $[Ca^{2+}]i$

GHRH is one of the primary neuropeptides stimulating GH release (Frohman et al., 1986). Through increased cAMP levels, GHRH is thought to convert voltage dependent Ca^{2+} channels from a non-functional to a functional form, which in turn causes a rapid increase in $[Ca^{2+}]i$, thereby promoting the release of GH via

exocytosis (Lussier, 1991). In this study, the resting $[Ca^{2+}]i$ of anterior pituitary cells was 155 +/- 10.4 nM (n = 4). Addition of GHRH (0.1 μM) caused a rapid, sustained increase in $[Ca^{2+}]i$, which plateaued at 186 +/- 11.5 nM and lasted for at least 10 minutes (Fig. 8).

c) Effect of Tg on $[Ca^{2+}]i$ in the presence of GHRH

This study determined whether the increase in $[Ca^{2+}]i$ by Tg and GHRH was additive or non-additive. The increase in $[Ca^{2+}]i$ by GHRH (0.1 μM) and Tg (20 μM) ($p < 0.05$; Fig. 9) was shown to be not significantly different than that by Tg alone ($p < 0.05$; Fig. 7). Neither the initial peak of $[Ca^{2+}]i$ nor the sustained level of $[Ca^{2+}]i$ were different than Tg's (20 μM) $[Ca^{2+}]i$ profile. Therefore, in the presence of Tg, GHRH did not further elevate $[Ca^{2+}]i$.

III.2 Effect of Tg on basal and GHRH-stimulated cAMP accumulation

The effect of the Ca^{2+}-mobilizer, Tg, on the GHRH-stimulated cAMP accumulation was examined. Treatment of rat anterior pituitary cells with GHRH (0.1 μM) caused a 55-fold increase in cAMP accumulation ($p < 0.05$; Fig. 10). Tg (20 μM) caused a small increase in basal cAMP accumulation, while lower concentrations of Tg had no effect. Addition of Tg increased the GHRH-stimulated cAMP accumulation in a concentration-dependent manner. At 20 μM, Tg

increased the GHRH-stimulated cAMP accumulation by 120% ($p < 0.05$; Fig. 10). Pretreatment with Tg for up to 150 minutes did not influence the effect of Tg on the GHRH-stimulated cAMP accumulation ($p < 0.05$; Table 3). Therefore, the anterior pituitary cells do not need to be pretreated with Tg because the effect of Tg on GHRH-stimulated cAMP accumulation is very rapid.

III.3 Effect of Tg on pituitary adenylate cyclase-activating polypeptide-, cholera toxin-, and forskolin-stimulated cAMP accumulation

To investigate the possible site(s) of action of Tg, pituitary adenylate cyclase- activating polypeptide (PACAP), another peptide hormone that also interacts with a G-protein coupled receptor in anterior pituitary cells (Canny et al., 1992), cholera toxin, which activates Gs (Cuttler et al., 1993), and forskolin, which directly activates adenylate cyclase (Downs et al., 1991), were used to stimulate cAMP accumulation. Treatment with PACAP (0.1 μM) caused a 5-fold increase in basal cAMP accumulation ($p < 0.05$; Table 4). Tg (20 μM) further increased the PACAP-stimulated cAMP accumulation 75% (Table 4). Treatment with cholera toxin (20 μg/ml) or forskolin (1 μM) increased cAMP accumulation by 6- and 21-fold, respectively ($p < 0.05$; Table 4). Tg remained effective in enhancing the cholera toxin- and forskolin-stimulated cAMP responses by 68% and 155%, respectively ($p < 0.05$; Table 4). These observations suggest that the effect of Tg is not limited to the GHRH-stimulated cAMP accumulation, and that the adenylate

cyclase may be one possible site of action for Tg.

III.4 Effect of Tg on cholera toxin- and forskolin-stimulated cAMP accumulation in GH_3 cells

Since the anterior pituitary consists not only of somatotrophs, but a variety of cell types, a more homogeneous cell type, the pituitary-derived GH_3 cells, were used. Cholera toxin (20 μg/ml) and forskolin (1 μM) both increased basal cAMP levels by 17-fold ($p < 0.05$; Table 5). Tg (20 μM) potentiated the cholera toxin-stimulated cAMP accumulation by 100% and the forskolin-stimulated cAMP accumulation by 150%, respectively ($p < 0.05$; Table 5). These results suggest that Tg is effective in potentiating both the cholera toxin- and forskolin-stimulated cAMP accumulation in a more homogeneous cell type, GH_3 cells, at the level of adenylate cyclase.

III.5 Effect of Tg on GHRH-stimulated cAMP accumulation in the presence of maximal phosphodiesterase inhibition

To examine the point of interaction between Tg and the GHRH-stimulated cAMP accumulation, the effect of Tg was determined in the presence of a phosphodiesterase inhibitor, IBMX. IBMX increased basal and GHRH-stimulated cAMP accumulation 6- and 2.4-fold, respectively. In the presence of IBMX, Tg

remained effective in potentiating the GHRH-stimulated cAMP accumulation by 23% ($p < 0.05$; Fig. 11), indicating that the effect of Tg is not due to the inhibition of the phosphodiesterase and that Tg may potentiate cAMP at a point upstream of the phosphodiesterase. The small increase in basal cAMP by Tg also persisted in IBMX-treated cells ($p < 0.05$; Fig. 11).

III.6 Effect of Ca^{2+}-chelators, EGTA and BAPTA-AM, on $[Ca^{2+}]i$ and cAMP accumulation

a) Effect of Tg on $[Ca^{2+}]i$ in the presence of the Ca^{2+} chelator, EGTA

The effect of Tg on $[Ca^{2+}]i$ was examined in the presence of EGTA, a Ca^{2+}-chelator. EGTA, in high concentrations, has been shown to "leach" $[Ca^{2+}]i$ out of essential intracellular Ca^{2+} pools in anterior pituitary cells (Lussier et al., 1988). In the presence of EGTA (0.5 mM), basal $[Ca^{2+}]i$ decreased to 60 +/- 1.4 nM ($n = 4$) (Fig. 12). EGTA also inhibited the large and rapid increase of $[Ca^{2+}]i$ evoked by Tg. In addition to inhibiting the initial rapid rise in $[Ca^{2+}]i$, EGTA inhibited the secondary sustained elevated $[Ca^{2+}]i$ evoked by Tg. This suggests that EGTA inhibits the $[Ca^{2+}]i$ elevating effect by Tg.

b) Effect of Tg on GHRH-stimulated cAMP accumulation in the presence of the Ca^{2+}-chelator, EGTA

To determine if the potentiating effect of Tg on the GHRH-stimulated cAMP accumulation was dependent on the elevation of $[Ca^{2+}]i$, EGTA, a Ca^{2+} chelator, was used. EGTA (0.5 mM) was effective in abolishing the increase in $[Ca^{2+}]i$ by Tg as shown in Fig. 12. Under this condition, the effect of Tg on GHRH-stimulated cAMP accumulation was reduced by 63% ($p < 0.05$; Fig. 13), indicating that the effect of Tg on cAMP is mediated through the elevation of $[Ca^{2+}]i$. Treatment with EGTA alone also enhanced basal- and GHRH-stimulated cAMP accumulation by 136% and 71%, respectively ($p < 0.05$; Fig. 13), suggesting that $[Ca^{2+}]i$ may have a dual effect on GHRH-stimulated cAMP accumulation with basal $[Ca^{2+}]i$ inhibiting the GHRH-stimulated cAMP accumulation, and high levels of $[Ca^{2+}]i$ potentiating the GHRH-stimulated cAMP accumulation.

c) Effect of Tg on $[Ca^{2+}]i$ in the presence of the intracellular Ca^{2+} chelator, BAPTA-AM

The effect of Tg on $[Ca^{2+}]i$ was examined in the presence of the membrane permeable, intracellular Ca^{2+}-chelator, BAPTA-AM. In the presence of BAPTA-AM (0.1 mM), basal $[Ca^{2+}]i$ decreased to 154 +/- 16.7 nM ($n = 4$) ($p < 0.05$; Fig. 14). BAPTA-AM also inhibited the large and rapid increase of $[Ca^{2+}]i$ by Tg (Fig. 14).

In addition, a gradual increase of $[Ca^{2+}]i$ was observed, which plateaued at 261 +/- 27.5 nM (n = 4), 2 min after Tg treatment. These results suggest that BAPTA-AM, reduced the large, rapid surge in $[Ca^{2+}]i$, and significantly suppressed the gradual increase in $[Ca^{2+}]i$ ($p < 0.05$; Fig. 14).

d) Effect of Tg on forskolin-stimulated cAMP accumulation in the presence of the intracellular Ca^{2+}-chelator, BAPTA-AM

To determine if the effect of Tg on the forskolin-stimulated cAMP accumulation was dependent on elevation of $[Ca^{2+}]i$, BAPTA-AM, an intracellular Ca^{2+} chelator, was used. Pretreatment with BAPTA-AM (0.1 mM) for 10 min was effective in abolishing the increase in $[Ca^{2+}]i$ by Tg as shown in Fig. 14. Under this condition, the effect of Tg on forskolin-stimulated cAMP accumulation was reduced by 75% ($p < 0.05$; Fig. 15), indicating that the effect of Tg on cAMP is mediated through the large, rapid surge in $[Ca^{2+}]i$. Pretreatment with BAPTA-AM alone also enhanced basal- and forskolin-stimulated cAMP accumulation by 76% and 53%, respectively ($p < 0.05$ for both; Fig. 15), again suggesting that $[Ca^{2+}]i$ may have a dual effect agonist-stimulated cAMP accumulation.

III.7 Effect of Tg on GHRH- or forskolin-stimulated cAMP accumulation in the presence of protein kinase inhibitors

Activation of protein kinase C, a Ca^{2+}-dependent enzyme, has been shown to enhance the GHRH-stimulated cAMP accumulation (French et al., 1989). To examine the possible involvement of protein kinase C on the Tg potentiation of GHRH-stimulated cAMP accumulation, H7, a non-specific protein kinase C inhibitor, and calphostin C, a more specific inhibitor of protein kinase C, were used. Neither H7 (0.1 mM) nor calphostin C (0.1 μM) alone had an effect on basal cAMP accumulation (data not shown). As shown in Table 6, H7 had no effect on the Tg potentiation of the GHRH-stimulated cAMP accumulation. Similarly, calphostin C also did not affect the Tg potentiation of the forskolin-stimulated cAMP accumulation (Table 6).

III.8 Effect of W7 on the Tg potentiation of GHRH-stimulated cAMP accumulation

The possible involvement of Ca^{2+}/calmodulin on the increasing effect of Tg on GHRH-stimulated cAMP accumulation was determined using W7, a specific inhibitor of Ca^{2+}/calmodulin dependent protein kinases (Schettini et al., 1984). GHRH (0.1 μM) increased basal cAMP accumulation by 65-fold ($p < 0.05$; Fig. 16). W7 (75 μM), which had no effect on basal or the GHRH-stimulated cAMP

accumulation ($p < 0.05$; Fig. 16), abolished the potentiating effect of Tg on the GHRH-stimulated cAMP accumulation ($p < 0.05$; Fig. 16), suggesting that the potentiation of the GHRH-stimulated cAMP accumulation by Tg is mediated through a Ca^{2+}/calmodulin dependent mechanism.

III.9 Effect of K^+ on $[Ca^{2+}]i$ and cAMP accumulation

a) Effect of K^+ on $[Ca^{2+}]i$

K^+, in depolarizing concentrations, has been shown to elevate $[Ca^{2+}]i$ in a variety of cell types, including anterior pituitary cells (Lussier, 1988). In this study, the effect of K^+ on $[Ca^{2+}]i$ in rat anterior pituitary cells was examined using fura-2 AM, so that K^+ could be compared to the $[Ca^{2+}]i$ elevation evoked by the intracellular Ca^{2+}-mobilizer, Tg. The resting $[Ca^{2+}]i$ of anterior pituitary cells was 189 +/- 12.9 nM (n = 4). Addition of K^+ (30 mM) caused an increase of $[Ca^{2+}]i$ to a sustained level of 248 +/- 8.6 nM (n = 4) (Fig. 17).

b) Effect of K^+ on GHRH-stimulated cAMP accumulation

To determine if other Ca^{2+} elevating agents had a similar potentiating effect on cAMP accumulation similar Tg, a depolarizing concentration of K^+ was used. K^+, as shown in Fig. 17, was shown to elevate $[Ca^{2+}]i$ in anterior pituitary

somatotrophs, by inducing the influx of Ca^{2+} across the plasma membrane (Lussier et al., 1988). K^+ (30 mM) was shown to inhibit basal levels of cAMP accumulation by a small, yet significant amount ($p < 0.05$; Fig. 18). K^+ (30 mM) was shown to inhibit the GHRH-stimulated cAMP accumulation by 85% ($p < 0.05$; Fig. 18). Thus, the two Ca^{2+} elevating agents, Tg and K^+, were shown to have very different effects on the GHRH-stimulated cAMP accumulation.

III.10 GHRH- and FSK-stimulated cAMP accumulation in the presence of lower concentration ranges of Tg

Since K^+ was shown to have inhibitory effects on the GHRH-stimulated cAMP pathway, and K^+ (30 mM) was shown to increase $[Ca^{2+}]i$ to lower elevations than Tg (20 μM), the possibility that different concentrations of $[Ca^{2+}]i$ effect adenylate cyclase activity differently was examined. To determine if Tg may have a dual effect on adenylate cyclase activity, the effect of Tg on the GHRH-, PACAP-, and forskolin-stimulated cAMP accumulation was investigated over a wider range of concentrations. Tg, 20 μM, was shown to enhance the GHRH-stimulated cAMP accumulation by 92%; while Tg, 0.02 μM, inhibited the GHRH-stimulated cAMP accumulation by 43% ($p < 0.05$; Table 7). Also, Tg, 20 μM, was found to potentiate the forskolin-stimulated cAMP accumulation by 81% ($p < 0.05$; Table 7), while Tg, 0.2 μM, was found to inhibit the forskolin-stimulated cAMP response by 45% ($p < 0.05$; Table 7). Finally, Tg, 20 μM and 2 μM, were found to increase the

PACAP-stimulated cAMP accumulation by 75% and 71%, respectively, while Tg (0.02 μM) was shown to inhibit the PACAP-stimulated cAMP levels by 54% ($p < 0.05$; Table 7).

III.11 Effect of Tg, K^+, and GHRH on GH release

Increases in $[Ca^{2+}]i$ have been shown to release GH via the process of exocytosis (Lussier et al., 1991). This study examined the effect of Tg, K^+, and GHRH on GH release from rat anterior pituitary somatotrophs. All three Ca^{2+} elevating agents were expected to and were found to increase the release of GH. Basal levels of GH released from the rat anterior pituitary somatotrophs were found to be 94.3 +/- 38.9 ng/well ($n = 4$). Tg (20 μM) increased GH release by 3.5-fold, while K^+ (30 mM) increased the GH release by 3-fold ($p < 0.05$; Fig. 20). GHRH (0.1 μM), the primary neuropeptide responsible for the release of GH, increased basal GH release by 2.8-fold ($p < 0.05$; Fig. 20). Tg (20 μM) increased the GHRH-stimulated GH release by 65%, while K^+ (30 mM) increased GHRH-stimulated GH release by 25% ($p < 0.05$; Fig. 20).

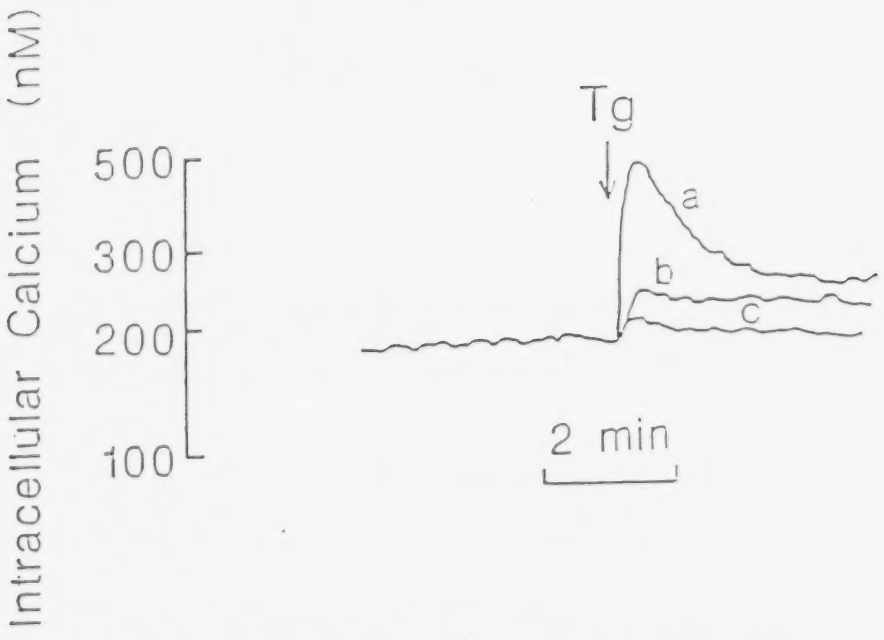

Figure III-1. Effect of thapsigargin (Tg) on $[Ca^{2+}]i$ in anterior pituitary cells. Cells were loaded with fura-2 and treated with various concentrations of Tg (a = 20 μM, b = 2 μM, and c = 0.2 μM). Ratio of the fluorescence emission signal at 510 nm with the excitation wavelengths set at 340 and 380 nm was continually recorded and calibrated as described. The tracing is representative of at least 3 experiments.

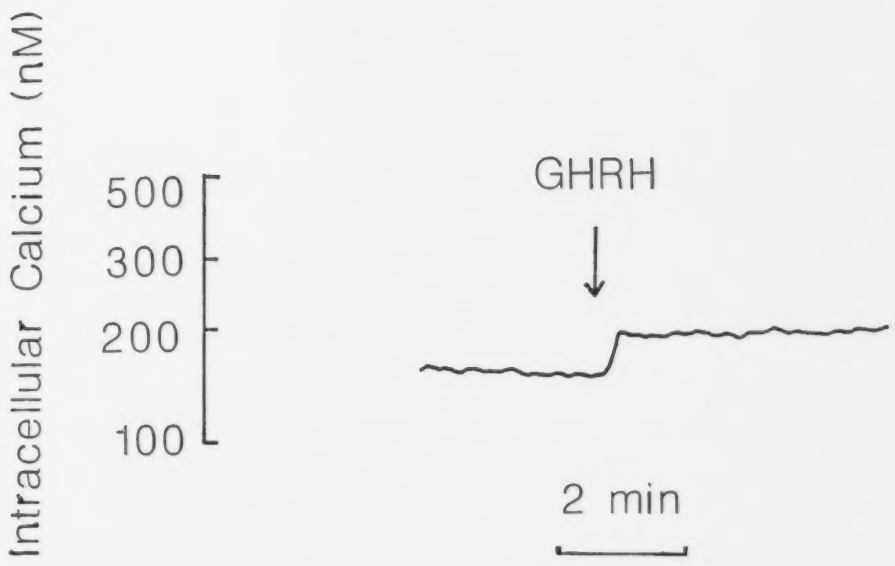

Figure III-2. Effect of GHRH on $[Ca^{2+}]i$ in anterior pituitary cells. Cells were loaded with fura-2 and treated GHRH (0.1 μM). Ratio of the fluorescence emission signal at 510 nm with the excitation wavelengths set at 340 and 380 nm was continually recorded and calibrated as described. The tracing is representative of at least 3 experiments.

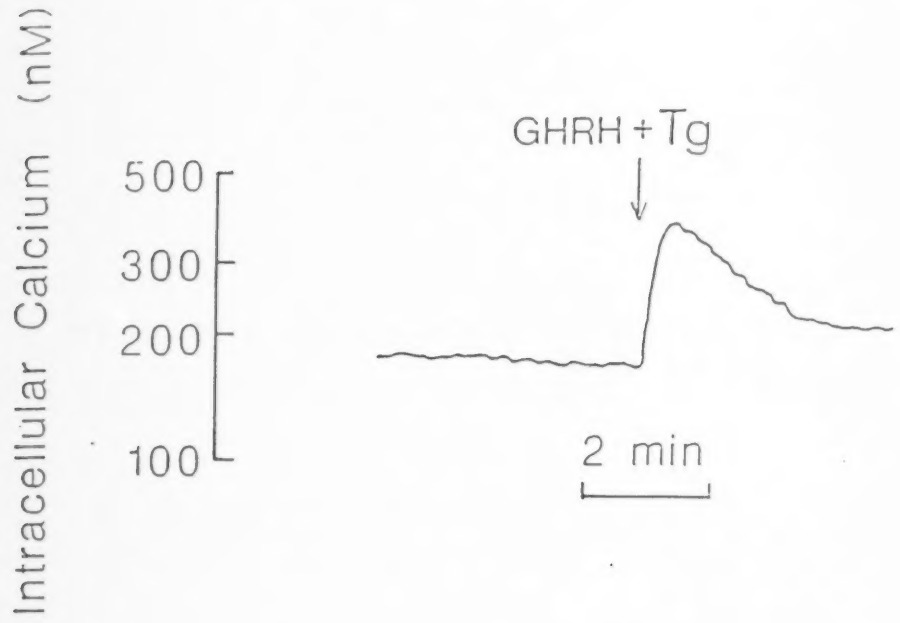

Figure III-3. Effect of GHRH and Tg on $[Ca^{2+}]i$ in anterior pituitary cells. Cells were loaded with fura-2 and treated with Tg (20 μM) and GHRH (0.1 μM). Ratio of the fluorescence emission signal at 510 nm with the excitation wavelengths set at 340 and 380 nm was continually recorded and calibrated as described. The tracing is representative of at least 3 experiments.

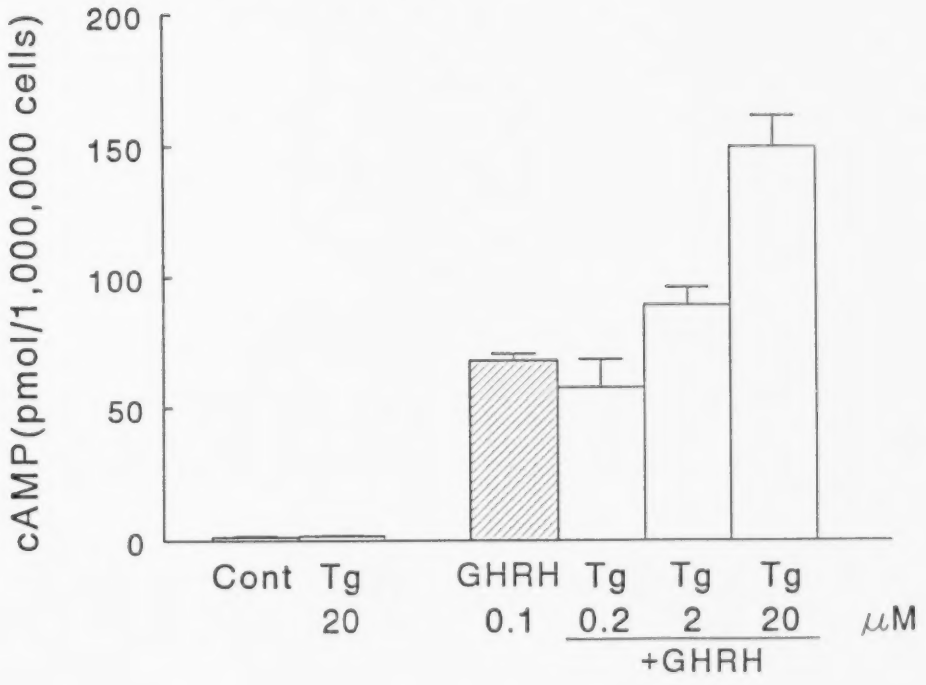

Figure III-4. Effect of Tg on GHRH-stimulated cAMP accumulation. Rat anterior pituitary were incubated with GHRH (0.1 μM) in the presence of graded concentrations of Tg. At the end of 15 min, the cellular cAMP content was determined. Each point represents the mean +/- SEM of cAMP determinations on four samples of cells.

Table I-3. Effect of duration of pretreatment with Tg on GHRH-stimulated cAMP accumulation

Treatment	Duration of Pretreatment with Tg (min)	cAMP (pmoles/10^6 cells)
Control	-	1.31 +/- 0.23
GHRH (100 nM)	-	52.17 /- 6.82
GHRH (100 nM) + Tg (1 μM)	0	74.48 +/- 2.30*
GHRH (100 nM) + Tg (1 μM)	5	73.86 +/- 7.67*
GHRH (100 nM) + Tg (1 μM)	30	76.32 +/- 2.34*
GHRH (100 nM) + Tg (1 μM)	60	82.52 +/- 11.89*
GHRH (100 nM) + Tg (1 μM)	150	81.41 +/- 5.86*

Each value represents the mean +/- SEM of cAMP determination done on four samples of cells.

* Significantly different from treatment with GHRH (100 nM); $p < 0.05$. There was no difference in cAMP accumulation among the groups with different duration of pretreatment.

Table I-4. Effect of Tg on PACAP-, cholera toxin-, and forskolin-stimulated cAMP accumulation

Treatment	cAMP (pmol/ 10^6 cells)
Control	2.09 +/- 0.30
PACAP (0.1 μM)	10.40 +/- 0.66
PACAP (0.1 μM) + Tg (20 μM)	18.22 +/- 1.72*
Cholera toxin (20 μg/ml)	13.32 +/- 1.22
Cholera toxin (20 μg/ml) + Tg (20 μM)	22.35 +/- 0.79*
Forskolin (1 μM)	44.05 +/- 1.75
Forskolin (1 μM) + Tg (20 μM)	113.50 +/- 14.95*

Each value represents the mean +/- SEM of cAMP determination done on four samples of cells.

* Significantly different from the corresponding treatment without Tg; $P < 0.05$.

Table I-5. Effect of Tg on cholera toxin- and forskolin-stimulated cAMP accumulation in GH_3 cells

Treatment	cAMP (pmol/10^6 cells)
Control	3.93 +/- 0.38
Tg (20 μM)	4.15 +/- 0.24
Cholera toxin (20 μg/ml)	68.09 +/- 4.70
Cholera toxin (20 μg/ml) + Tg (20 μM)	129.38 +/- 9.72*
Forskolin (1 μM)	65.51 +/- 3.18
Forskolin (1 μM) + Tg (20 μ)	164.15 +/- 21.6*

Each value represents the mean +/- SEM of cAMP determination done on four samples of cells.

* Significantly different from the corresponding treatment without Tg; $P < 0.05$.

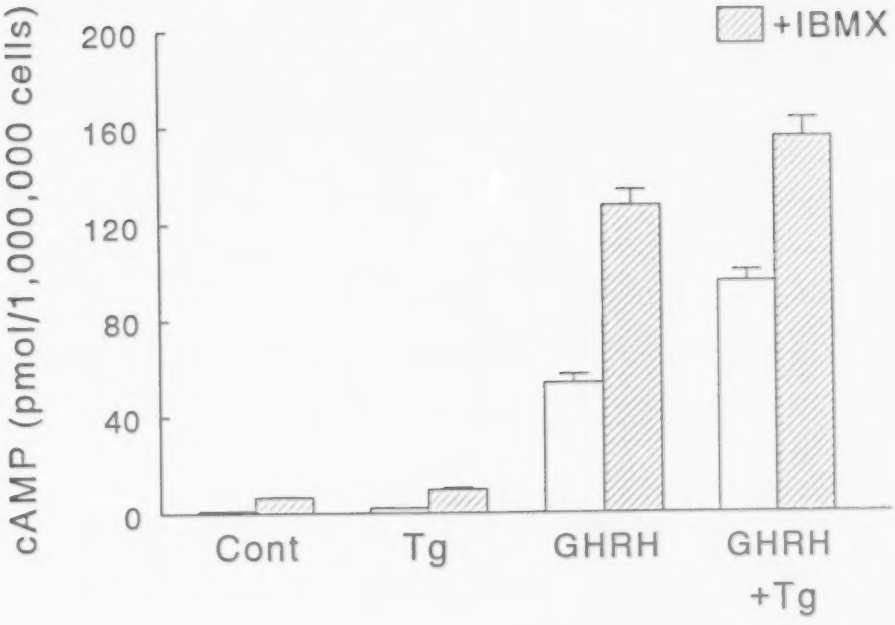

Figure III-5. Effect of Tg on GHRH-stimulated cAMP accumulation in the presence of maximal phosphodiesterase inhibition. Rat anterior pituitary cells were incubated with GHRH (0.1 μM) and Tg (20 μM) in the presence or absence of a phosphodiesterase inhibitor, IBMX (1 mM). At the end of 15 min, the cellular cAMP content was determined. Each point represents the mean +/- SEM of cAMP determinations on four samples of cells.

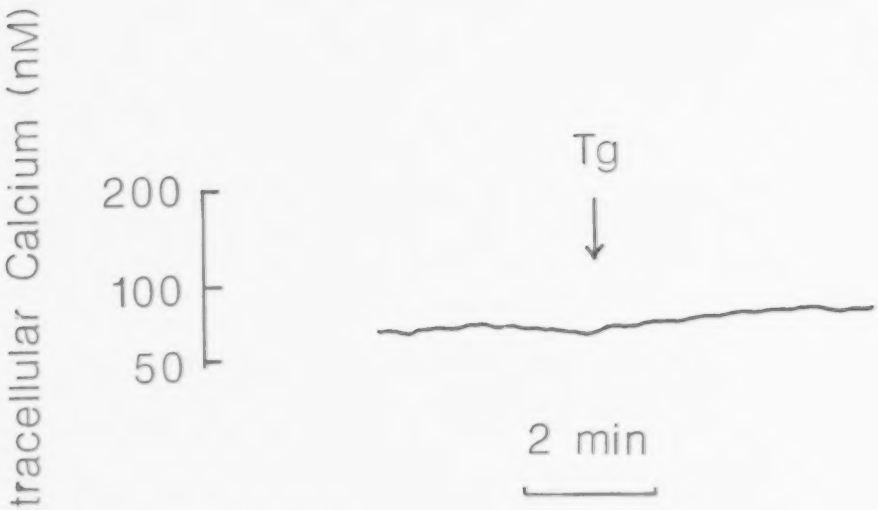

Figure III-6. Effect of Tg on $[Ca^{2+}]i$ pretreated with the Ca^{2+}-chelator, EGTA, in anterior pituitary cells. Cells were loaded with fura-2 and treated with Tg (20 μM) in the presence of EGTA (0.5 mM). Ratio of the fluorescence emission signal at 510 nm with the excitation wavelengths set at 340 and 380 nm was continually recorded and calibrated as described. The tracing is representative of at least 3 experiments.

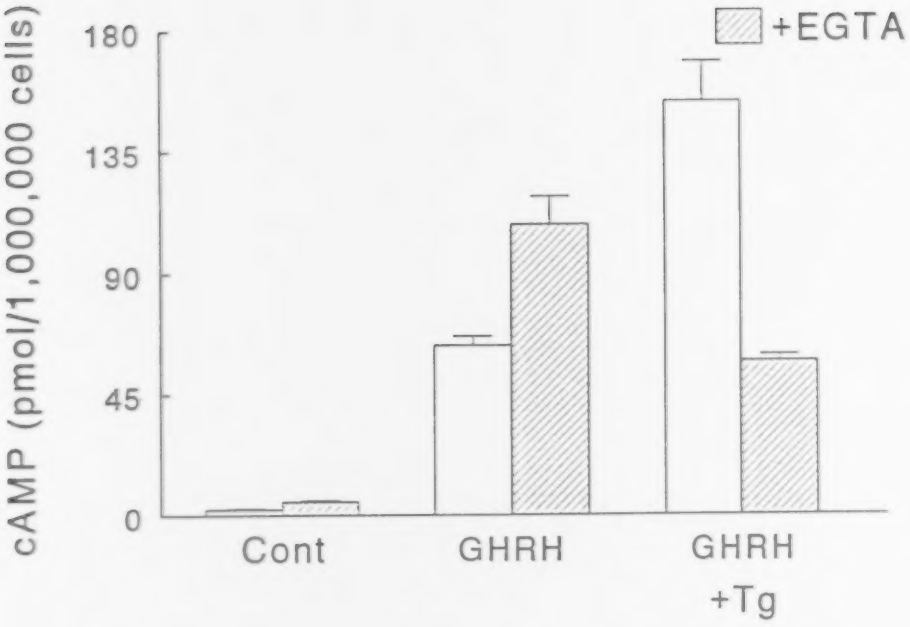

Figure III-7. Effect of Tg on GHRH-stimulated cAMP accumulation in the presence of the Ca^{2+}-chelator, EGTA. Rat anterior pituitary cells were incubated with GHRH (0.1 μM) and Tg (20 μM) in the presence or absence of the extracellular Ca^{2+} chelator, EGTA (0.5 mM). At the end of 15 min, the cellular cAMP content was determined. Each point represents the mean +/- SEM of cAMP determinations on four samples of cells.

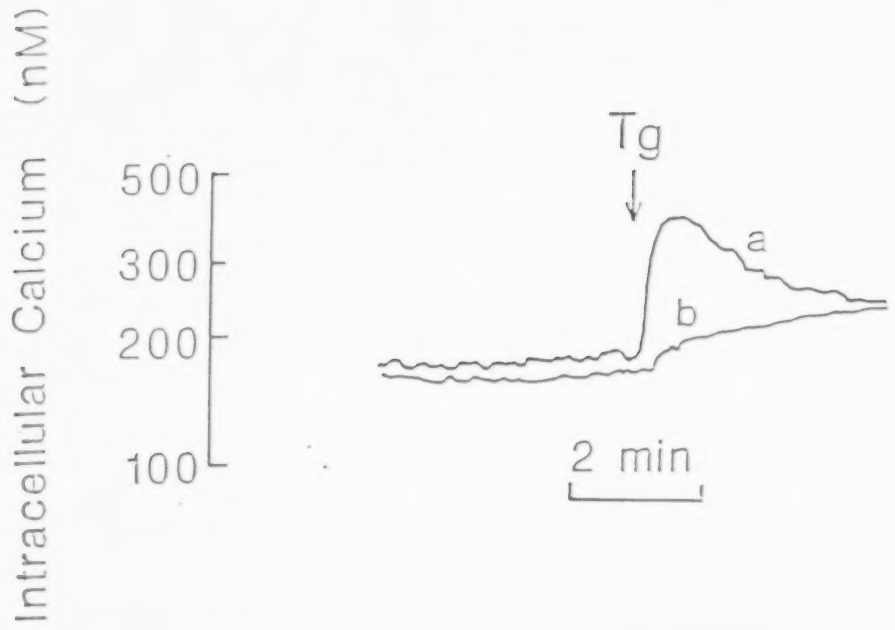

Figure III-8. Effect of Tg on $[Ca^{2+}]i$ in the presence of the intracellular Ca^{2+} chelator, BAPTA-AM, in anterior pituitary cells. Cells were loaded with fura-2 and treated with Tg (20 μM) in the presence or absence of BAPTA-AM (0.1 mM). BAPTA-AM required a 10 min pretreatment period (a = Tg; b = Tg + BAPTA-AM. Ratio of the fluorescence emission signal at 510 nm with the excitation wavelengths set at 340 and 380 nm was continually recorded and calibrated as described. The tracing is representative of at least 3 experiments.

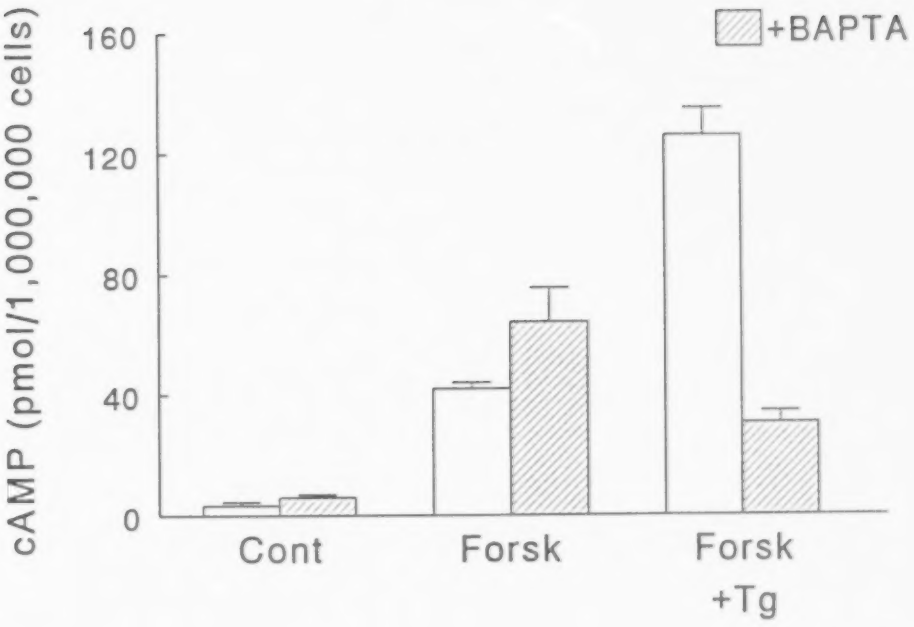

Figure III-9. Effect of Tg on forskolin-stimulated cAMP accumulation in the presence of the intracellular chelator, BAPTA-AM. Rat anterior pituitary cells were incubated with forskolin (1 μM) and Tg (20 μM) in the presence or absence of the intracellular Ca^{2+}-chelator, BAPTA (0.1 mM). BAPTA-AM required a 10 min pretreatment period. At the end of 15 min, the cellular cAMP content was determined. Each point represents the mean +/- SEM of cAMP determinations on four samples of cells.

Table I-6. Effect of calphostin C and H7 on the potentiation of Tg on GHRH- and forskolin-stimulated cAMP accumulation

Treatment	cAMP (pmoles/10^6 cells)
Experiment I	
Control	1.59 +/- 0.18
GHRH (100 nM)	30.96 +/- 3.45
GHRH (100 nM) + H7 (0.1 mM)	29.79 +/- 2.85
GHRH (100 nM) + Tg (20 μM)	55.95 +/- 6.15*
GHRH (100 nM) + H7 (0.1 mM) + Tg (20 μM)	52.50 +/- 7.02*
Experiment II	
Control	1.06 +/- 0.12
Forskolin (1 μM)	38.32 +/- 4.96
Forskolin (1 μM) + Calphostin C (1 μM)	33.64 +/- 2.30
Forskolin (1 μM) + Tg (20 μM)	86.38 +/- 7.66*
Forskolin (1 μM) + Calphostin C (1 μM) + Tg (20 μM)	97.42 +/- 12.10*

Each value represents the mean +/- SEM of cAMP determinations done on four samples of cells.

* Significantly different from treatment without Tg; $P < 0.05$.

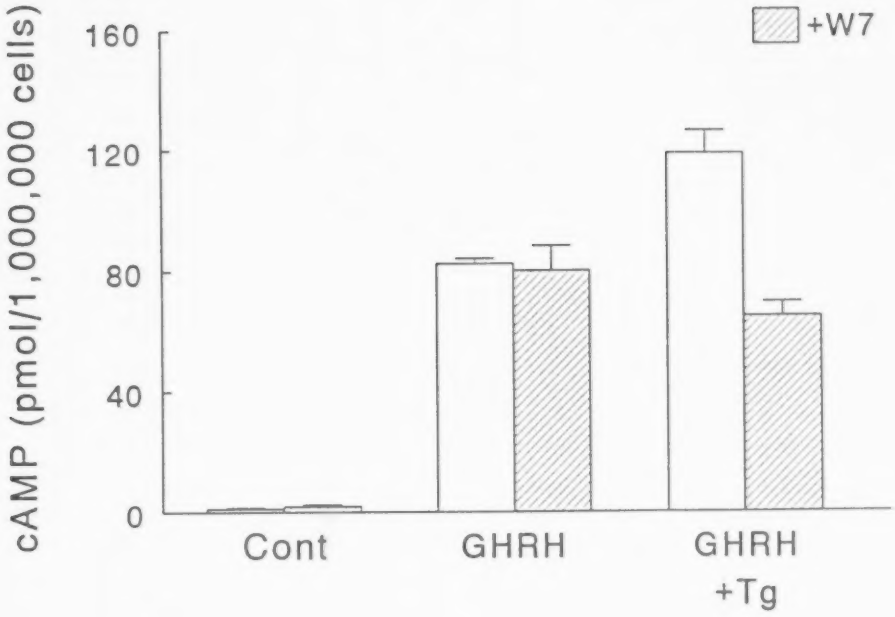

Figure III-10. Effect of Tg on GHRH-stimulated cAMP accumulation in the presence of a Ca^{2+}/calmodulin inhibitor, W7. Rat anterior pituitary cells were incubated with GHRH (0.1 μM) and Tg (20 μM) in the presence or absence of a Ca^{2+}/calmodulin inhibitor, W7 (75 μM). At the end of 15 min, the cellular cAMP content was determined. Each point represents the mean +/- SEM of cAMP determinations on four samples of cells.

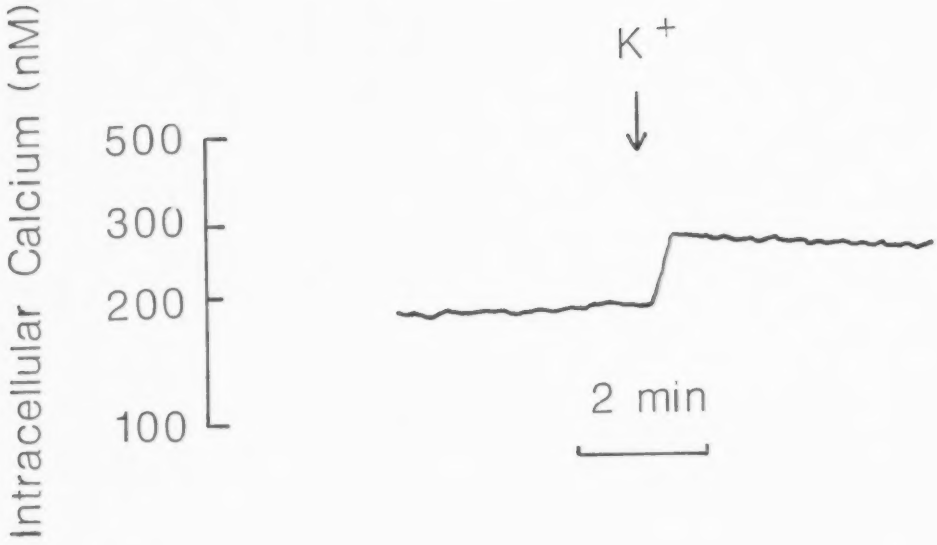

Figure III-11. Effect of K^+ on $[Ca^{2+}]i$ in anterior pituitary cells. Cells were loaded with fura-2 and treated with a depolarizing concentration of K^+ (30 mM). Ratio of the fluorescence emission signal at 510 nm with the excitation wavelengths set at 340 and 380 nm was continually recorded and calibrated as described. The tracing is representative of at least 3 experiments.

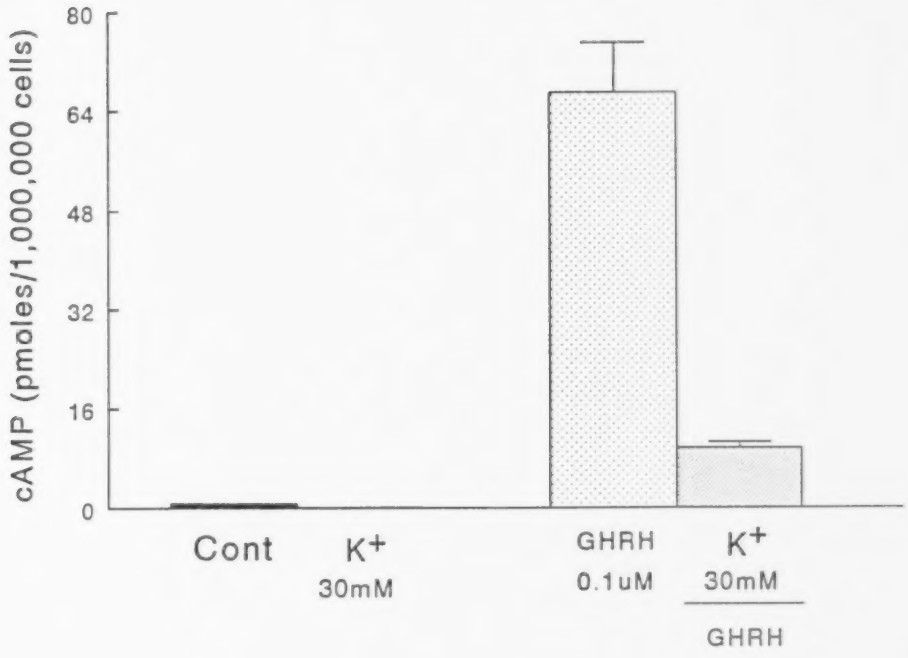

Figure III-12. Effect of K^+ on GHRH-stimulated cAMP accumulation. Rat anterior pituitary cells were incubated with and K^+ (30 mM) in the presence or absence of GHRH (0.1 μM). At the end of 15 min, the cellular cAMP content was determined. Each point represents the mean + /- SEM of cAMP determinations on four samples of cells.

Table I-7. GHRH-stimulated cAMP accumulation in the presence of lower concentrations of Tg

Treatment	cAMP (pmol/10^6 cells)
Experiment I	
Control	1.48 +/- 0.39
GHRH (100 nM)	78.23 +/- 1.51
GHRH + Tg (20 μM)	149.93 +/- 11.71*
GHRH + Tg (2 μM)	89.37 +/- 6.80
GHRH + Tg (0.2μM)	57.75 +/- 10.85
GHRH + Tg (0.02μM)	44.74 +/- 2.54+
Experiment II	
Control	0.53 +/- 0.16
Forskolin (1 μM)	26.72 +/- 1.21
Forskolin + Tg (20 μM)	48.26 +/- 5.18*
Forskolin + Tg (2 μM)	24.48 +/- 2.48
Forskolin + Tg (0.2 μM)	14.86 +/- 1.47+
Experiment III	
Control	0.71 +/- .005
PACAP (100 nM)	5.20 +/- 0.33
PACAP + Tg (20 μM)	9.11 +/- 0.86*
PACAP + Tg (0.2 μM)	8.88 +/- 1.03*
PACAP + Tg (0.02 μM)	2.37 +/- 0.67+

Each value represents the mean +/- SEM of cAMP determinations done on four samples of cells.

* Significantly higher than treatment with GHRH, FSK, or PACAP; $P < 0.05$.
+ Significantly lower than treatment with GHRH, FSK, or PACAP; $P < 0.05$.

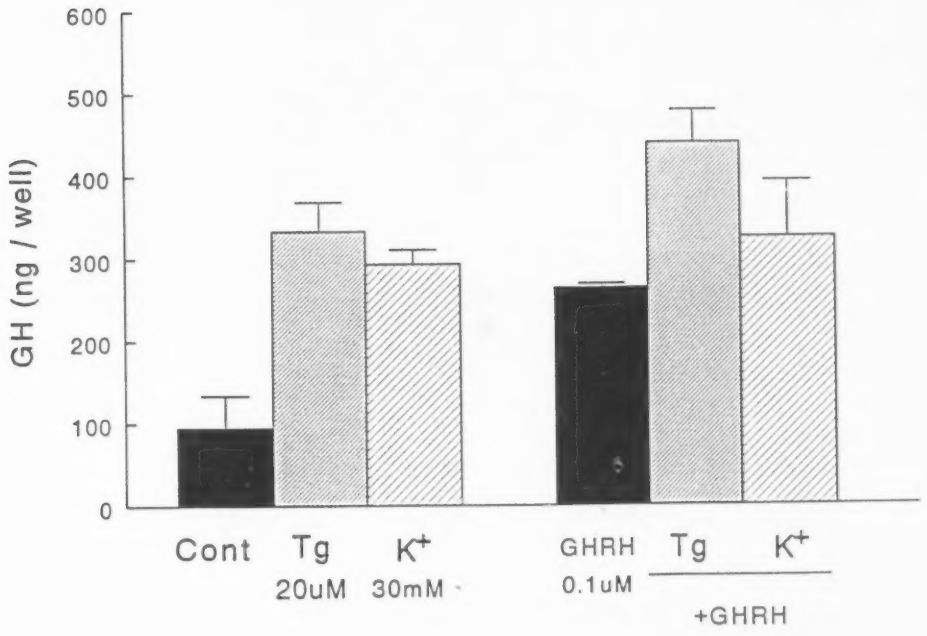

Figure III-13. Effect of Tg and K^+ on GH release. Rat anterior pituitary cells were incubated with Tg (20 μM) and K^+ (30 mM) in the presence or absence of GHRH (0.1 μM). At the end of 15 min, the medium was removed and assayed for GH levels. Each point represents the mean +/- SEM of GH determinations done in duplicated on four samples of cells.

Discussion

Intracellular Ca^{2+} has been long recognized as a potentially important modulator of the cAMP / adenylate cyclases system (Warhurst et al., 1994). Functional interactions between elevation of $[Ca^{2+}]i$ and adenylate cyclases have been reported in several studies (Amiranoff et al., 1983; Caldwell et al., 1992). Incubation of membranes from several cell types with micromolar concentrations of Ca^{2+} stimulates a significant increase in adenylate cyclase activity (Amiranoff et al., 1983; Caldwell et al., 1992). Indeed, more and more data suggest that Ca^{2+} can stimulate or inhibit particular Ca^{2+}-sensitive adenylate cyclase isoforms as, or more, effectively than G-protein α-subunits (Tang et al., 1992; Iyengar, 1993).

In this study, the interaction between Ca^{2+} and GHRH-stimulated cAMP accumulation in rat anterior pituitary cells was investigated using the Ca^{2+}-mobilizer, Tg. It was found that Tg potentiated the GHRH-stimulated cAMP accumulation in a concentration-dependent manner. Since Tg selectively elevates $[Ca^{2+}]i$ without promoting the breakdown of phosphatidyl inositols, the effect of Tg on the GHRH-stimulated cAMP accumulation was likely mediated by Ca^{2+}, either directly or indirectly through a Ca^{2+}-calmodulin dependent protein kinase, rather than through phosphatidylinositol turnover or translocation of protein kinase C (Thastrup, 1990; Houslay, 1991; van den Berghe et al., 1992). A similar effect on the GHRH-stimulated cAMP accumulation has been observed with the Ca^{2+}-

ionophore, A23187 (Schettini et al., 1984).

IV.1 Thapsigargin and intracellular Ca^{2+}

Tg's effect on $[Ca^{2+}]i$ in rat anterior pituitary cells is similar to its effect on other cell types, such as hepatocytes, platelets, and parotid acinar cells (Takemura et al., 1990; Thastrup et al., 1989). Previous studies, in these cell types, have shown that Tg mobilizes $[Ca^{2+}]i$ in two phases: (1) the early phase of $[Ca^{2+}]i$ derived from mainly the intracellular Ca^{2+} pool; (2) the secondary or sustained phase, which is dependent on extracellular Ca^{2+} moving across the plasma membrane (Takemura et al., 1990). In our study, Tg is also shown to increase $[Ca^{2+}]i$ biphasically. There was first a rapid, initial increase from 189 to 460 nM, followed by a sustained increase in $[Ca^{2+}]i$ (263 nM). The first phase was presumably due to the inhibition of the 1,4,5-IP3 sensitive ER Ca^{2+}-ATPase by Tg, which was immediately followed by the efflux of Ca^{2+} through "leakage" channels on the membrane of the ER. Pretreatment with the intracellular Ca^{2+} chelator, BAPTA, blocked the initial, rapid increase in $[Ca^{2+}]i$ by Tg. The second phase was probably due to Ca^{2+} influx across the plasma membrane triggered by the emptying of the intracellular Ca^{2+}-stores (possibly due to Ca^{2+}-mediated Ca^{2+} channels). The Ca^{2+} chelator, EGTA, was found to inhibit the second or sustained phase of Ca^{2+}-entry. These experiments provide support for the most fundamental concept of the "capacitance Ca^{2+}-entry model", which states that the Ca^{2+} content

of agonist- and 1,4,5-IP3-regulated intracellular Ca^{2+} pool is the primary determinant of the rate of Ca^{2+} entry across the plasma membrane (Putney, 1986). In other words, it seems that, in anterior pituitary cells, Tg acts as a useful pharmacological marker in understanding Ca^{2+} influx which adheres to the capacitative entry model. Our proposed model of Ca^{2+}-entry from the extracellular space appears to be a highly-regulated Ca^{2+}-signalling system through which the rate of entry of Ca^{2+} across the plasma membrane can be closely co-ordinated with the extent of depletion of intracellular Ca^{2+} stores, unlike some other cell types, such as NG115-401L neuronal cells (Jackson et al, 1988), in which Ca^{2+} entry in dependent on the formation of inositol phosphates.

IV.2 Intracellular Ca^{2+} elevation; the key to thapsigargin's effect

With respect to the mechanism through which Tg mediates its effect on GHRH- or agonist-stimulated cAMP accumulation, our results strongly indicate the involvement of increased $[Ca^{2+}]i$ levels. Tg has been reported to have Ca^{2+}-independent actions in rat anterior pituitary cells (Pepperell et al., 1990). However, pretreatment with the intracellular Ca^{2+} chelator, BAPTA, which, as stated previously, blocked the initial, rapid elevation of $[Ca^{2+}]i$, and was also effective in inhibiting the potentiating effect of Tg on agonist-stimulated cAMP accumulation, which suggests that the initial, rapid mobilization of Ca^{2+} from intracellular stores by Tg is necessary for its potentiation of the agonist-stimulated cAMP

accumulation. EGTA, the Ca^{2+}, chelator was also successful in attenuating the enhancement of agonist-stimulated cAMP response by Tg suggesting that extracellular Ca^{2+} may also be a necessary component of Tg's potentiating effect. Although some reported effects of Tg have been attributed solely to the depletion of intracellular Ca^{2+} stores (Ghosh et al., 1991), our results indicate that this is an unlikely explanation since the duration of pretreatment did not alter the effect of Tg.

IV.3 The site of interaction between thapsigargin and the agonist-stimulated cAMP pathway

An interesting aspect of the interaction between cAMP and Ca^{2+} in anterior pituitary cells is that Tg, which elevates $[Ca^{2+}]i$ within a physiologically relevant range, unlike many Ca^{2+} ionophores (Warhurst et al., 1994), appears to "sensitize" or increase the rate of agonist-stimulated cAMP synthesis. Several observations suggest that at least one site of interaction between Tg and the agonist-stimulated cAMP pathway is at the level of the adenylate cyclase. First, the potentiation by Tg of the GHRH- and PACAP-stimulated cAMP accumulation suggesting that Tg probably interacts with the cAMP signal transduction pathway downstream of the GHRH and PACAP receptors. The reason being is that GHRH receptors and PACAP receptors share common downstream components, such as the G_s-proteins and the adenylate cyclases, along the cAMP signal transduction pathway (Canny et al., 1992). However, there is a very small possibility that Tg interacts at

the receptor level for both the GHRH- and PACAP-stimulated cAMP accumulation because GHRH and PACAP receptors have been shown to share sequence homology.

The second line of evidence that Tg acts at the level of adenylate cyclase is that Tg remained effective in potentiating the cholera toxin- and forskolin-stimulated cAMP accumulation. This result was also shown in the pituitary-derived GH_3 tumour cells, which are a much more homogeneous population of cells than anterior pituitary cells. This suggests that Tg must act downstream of the G_s-protein, and that at least one point of interaction between Tg and the agonist-stimulated cAMP pathway must be the adenylate cyclase.

The third observation that strongly suggested that Tg acted at the level of adenylate cyclase was that the effect of Tg persisted in the presence of maximal phosphodiesterase inhibition using IBMX, suggesting that the effect of Tg on agonist-stimulated cAMP accumulation is mediated through increased synthesis of cAMP through adenylate cyclase activation rather than an inhibition of the hydrolysis of cAMP, which is mediated through phosphodiesterase activation.

IV.4 Calmodulin; an intracellular mediator of thapsigargin's effect

Protein kinase C has been implicated as an important modulator in several cell types (Warhurst et al., 1994). Protein kinase C was shown to potentiate basal- and agonist-stimulated cAMP accumulation in GH_3 pituitary cells (Quilliam et al., 1989), β-lymphocytes (Weiner et al., 1989), and rat pinealocytes (Sugden et al., 1985). Also, elevation of $[Ca^{2+}]i$ was shown to activate protein kinase C and protein kinase C activators have been shown to increase GHRH-stimulated cAMP accumulation (French et al., 1989). Tg, as stated before, selectively raises $[Ca^{2+}]i$ without activating phosphatidyl inositol or translocation of protein kinase C (Thastrup, 1990; van den Berghe et al., 1992). However, elevated $[Ca^{2+}]i$ has been shown to activate protein kinase C on its own, and Tg has been shown to increase protein kinase C, via elevated $[Ca^{2+}]i$, in some cell types, such as platelets (Thastrup et al., 1989). Therefore, to determine if Tg mediated its potentiating effect on agonist-stimulated cAMP accumulation via the activation of protein kinase C, protein kinase C inhibitors, H7 and calphostin C (a more specific protein kinase inhibitor), were used. Our results indicate that the potentiating effect of Tg is independent of protein kinase C activation. This is based on the observations that H7 and calphostin C do not affect the Tg potentiation of agonist-stimulated cAMP accumulation.

Although a direct effect of $[Ca^{2+}]i$ on adenylate cyclase activity has been

reported in several cell types (Cooper et al., 1995), our results strongly indicate that the action of Tg is mediated through a Ca^{2+}/calmodulin dependent protein kinase since W7, a specific Ca^{2+}/calmodulin inhibitor, abolished the potentiating effect of Tg on the agonist-stimulated cAMP accumulation. Indeed, it has been well established that the stimulation of types I, III, and VIII adenylate cyclases is mediated by calmodulin, which can be removed or added back, restoring Ca^{2+} sensitivity (Bakalyar et al., 1990; Yoshimura et al., 1992; Tang et al., 1992). It is most likely that Tg, via elevated $[Ca^{2+}]i$, activates the type I adenylate cyclase (Ca^{2+}/calmodulin dependent adenylate cyclase), since it has been shown that the concentration of Ca^{2+} that stimulates the type I adenylate cyclase is in the normal physiological range (0.1 - 1 μM), whereas the stimulation of types III and type VIII is by supra-physiological Ca^{2+} concentrations (> 1μM) (Yoshimura et al., 1992; Choi et al., 1992). Consistent with these observations is a previous report demonstrating that submicromolar Ca^{2+}-concentrations were stimulatory to the pituitary adenylate cyclase and that exogenously added calmodulin enhanced Ca^{2+} stimulation of the adenylate cyclase in pituitary cell membranes (Schettini et al., 1984). Other studies, by Warhurst et al. (1994), have shown in broken cell preparations that calmodulin in the presence of > 1 μM free Ca^{2+} caused stimulation of forskolin-activated adenylate cyclase (Warhurst et al., 1994). The precise mechanism through which Tg activates the Ca^{2+}/calmodulin dependent protein kinase, which in turn modulates the anterior pituitary adenylate cyclases remains to be investigated.

IV.5 Intracellular Ca^{2+} and agonist-stimulated cAMP accumulation

Another interesting observation, in the present study, is that pretreatment with BAPTA was found to enhance basal- and forskolin-stimulated cAMP accumulation suggesting that reducing $[Ca^{2+}]i$ could also enhance agonist-stimulated cAMP accumulation. EGTA, in our study, was also found to increase basal- and GHRH-stimulated cAMP accumulation. Increases in basal- and GHRH-stimulated cAMP accumulation has been previously observed with a Ca^{2+}-free medium or treatment with EGTA in anterior pituitary cells (Lussier et al., 1988). Therefore, Ca^{2+} appears to have a dual effect on agonist-stimulated cAMP accumulation. At $[Ca^{2+}]i$ elevations around basal level, $[Ca^{2+}]i$ inhibits basal- and agonist-stimulated cAMP accumulation, and higher concentrations of $[Ca^{2+}]i$ potentiating basal- and agonist-stimulated cAMP accumulation. This biphasic effect of Ca^{2+} on adenylate cyclase activity has also been observed in studies using several cell-free systems (Warhurst et al., 1994; Amiranoff et al., 1983; Caldwell et al., 1992; MacNeil et al., 1985).

There are various possible explanations for the dual effect of $[Ca^{2+}]i$ on cAMP accumulation in rat anterior pituitary cells. It is possible that at basal or slightly elevated levels of $[Ca^{2+}]i$ that there is no activation of the Ca^{2+}-calmodulin dependent kinase. Therefore, the Ca^{2+}/calmodulin dependent adenylate cyclase is not activated. However, at these levels of basal or slightly elevated $[Ca^{2+}]i$

levels, such as with K^+ (30 mM) or Tg (lower concentration ranges such as 0.2 or 0.02 μM), it is possible that $[Ca^{2+}]i$ activates a Ca^{2+}/calmodulin dependent phosphodiesterase, thereby decreasing agonist-stimulated cAMP accumulation (Conti et al., 1991). A further possibility is that, at basal or slightly elevated $[Ca^{2+}]i$, there is a Ca^{2+}-dependent inhibition of adenylate cyclase activity (adenylate cyclase V or VI, which are Ca^{2+}-inhibitable adenylate cyclases isoforms), in which calmodulin does not seem to be mediating this inhibitory effect (Yoshimura et al., 1992; Boyajian et al., 1991). This inhibition of adenylate cyclase activity by Ca^{2+} could also account for the decrease in cAMP accumulation. Therefore, when BAPTA and EGTA are added, the decreased basal $[Ca^{2+}]i$ enhances cAMP accumulation by removing the inhibitory effect of basal or slightly elevated levels of $[Ca^{2+}]i$ on a Ca^{2+}-inhibitable adenylate cyclase isoform or a Ca^{2+}/calmodulin dependent phosphodiesterase, thus increasing cAMP accumulation. This inhibition of agonist-stimulated cAMP accumulation by lower elevations of $[Ca^{2+}]i$ has been reported in anterior pituitary cells (Narayanan et al., 1989) and in other cell types (Yu et al., 1993).

In comparison, a large elevation of $[Ca^{2+}]i$, such as that mobilized by Tg or A23187 (Schettini, 1984), potentiates the adenylate cyclase pathway by activating a Ca^{2+}-calmodulin dependent adenylate cyclase isoform (Type I, III, or VIII adenylate cyclase isoforms), and thus increasing the rate of synthesis of cAMP. Another possibility could be that $[Ca^{2+}]i$ at all concentrations activates a

Ca^{2+}/calmodulin dependent phosphodiesterase or Ca^{2+}-inhibitable adenylate cyclase isoform, thereby decreasing cAMP accumulation, and only at higher levels of $[Ca^{2+}]i$, such as that evoked by Tg, is the rate of activation of the Ca^{2+}/calmodulin dependent adenylate cyclase greater than the rate of hydrolysis of cAMP caused by the Ca^{2+}/calmodulin dependent phosphodiesterase or the inhibition of cAMP formation caused by a Ca^{2+}-inhibitable adenylate cyclase isoform (Type V or VI adenylate cyclase isoforms), thus resulting in an increase in basal- and agonist-stimulated cAMP accumulation.

Indeed, it has been shown in various studies that there are multiple adenylate cyclase isoforms expressed in one cell type (Yu et al., 1993) and there is an optimal concentration of $[Ca^{2+}]i$ needed for activation of each of the different adenylate cyclase isoforms (Cooper et al., 1995; Ishikawa et al., 1992) and activation of the Ca^{2+}-calmodulin dependent phosphodiesterase (Conti et al., 1991). An interesting characteristic of some of the adenylate cyclase isoforms is that, in some cell types, the Ca^{2+}-inhibitable adenylate cyclase isoforms are inhibited in a similar concentration range that Ca^{2+} activates the Ca^{2+}/calmodulin adenylate cyclase isoforms (Yu, 1993). Therefore, we suggest that the regulation of cAMP accumulation by $[Ca^{2+}]i$ in rat anterior pituitary cells is very complicated because there may be different isoforms of the adenylate cyclase present, which are activated and inhibited by different elevations of $[Ca^{2+}]i$. The situation is further complicated by the possible role of Ca^{2+} in activating the Ca^{2+}/calmodulin

dependent phosphodiesterase, thus promoting the hydrolysis of cAMP (Fig. 20).

IV.6 Intracellular Ca^{2+} and growth hormone release

It is expected that vesicle or peptide hormone release from most secretagogues is dependent on Ca^{2+} (Schettini, 1984), therefore it came as no surprise that Tg and K^+ both increased the basal- and GHRH-stimulated GH release due to increased $[Ca^{2+}]i$ levels. Tg slightly enhanced GH release more than K^+. However, these results should be viewed with caution because it is very difficult to differentiate between the direct effects of $[Ca^{2+}]i$ on growth hormone release and the indirect effects of $[Ca^{2+}]i$ on growth hormone release, such as the potentiation or inhibition of cAMP accumulation.

IV.7 Summary

In summary, the synergistic interaction between the Ca^{2+}-signalling pathway and agonist-stimulated cAMP accumulation in rat anterior pituitary cells was characterized using Tg. Tg, as a novel pharmacological tool, allowed us to examine the interaction of $[Ca^{2+}]i$ and agonist-stimulated cAMP accumulation, with minimal interference of other signal transduction pathways. This study raised the possibility of multiple mechanisms, which appear to be involved in the interaction between these two signalling systems. In addition to the possible direct effects of

Ca^{2+} on cAMP metabolism, such as a Ca^{2+}-inhibitable adenylate cyclase isoform, a Ca^{2+}/calmodulin dependent protein kinase also appears to be involved in the increased rate of activity of a Ca^{2+}/calmodulin-stimulated adenylate cyclase isoform, thus promoting the increase in cAMP accumulation. A Ca^{2+}/calmodulin dependent protein kinase may also be involved in the activation of a phosphodiesterase. The interaction between different levels of $[Ca^{2+}]i$ and the GHRH- and other agonist-stimulated cAMP accumulation could play a crucial role in the understanding of the regulation of growth hormone release in anterior pituitary somatotrophs, and hormone secretion in many other cell types.

SUMMARY

Treatment	$[Ca^{2+}]i$	AC or PDE activity	cAMP accumulation
Tg (20 uM)	High (> 300 nM)	stimulates AC (Type I, III, VIII AC)	increased
K^+ (30 mM)	Basal or slightly elevated (150 nM - 300 nM)	inhibits AC (Type V or VI AC)	decreased
Tg (0.2 uM) Tg (0.02 uM)		stimulates PDE	
Control			
BAPTA (0.1 mM)	Low (< 150 nM)	stimulates AC (Type V or VI AC)	increased
EGTA (0.5 mM)		inhibits PDE	

Figure IV-1. Summary: hypothetical model for regulation of GHRH-stimulated cAMP accumulation by $[Ca^{2+}]i$.

V. Future studies

a) Effects of Ca^{2+} and calmodulin on adenylate cyclase activity and phosphodiesterase activity

It has been determined that Tg, via the elevation of $[Ca^{2+}]i$, potentiates the GHRH-stimulated cAMP accumulation and also stimulates GH release from rat anterior pituitary cells. The exact point and mechanism of interaction between $[Ca^{2+}]i$, or a Ca^{2+}/calmodulin dependent protein kinase, and the cAMP signal transduction pathway (hormone-receptor-G_s protein-adenylate cyclase) remains to be determined. A study of the effects of Ca^{2+}, the Ca^{2+}/calmodulin complex, and Tg, by itself, on the pituitary adenylate cyclase and the pituitary phosphodiesterase would likely prove to be a useful study.

b) Isolation and purification of adenylate cyclase isoforms in anterior pituitary cells

It has been determined that it is likely that Tg, via a Ca^{2+}/calmodulin dependent protein kinase, activates an adenylate cyclase. K^+ was shown to inhibit cAMP accumulation, possibly via a Ca^{2+}-inhibitable adenylate cyclase isoform. It would be a useful study to isolate and purify different adenylate cyclase isoforms in anterior pituitary cells to explain the biphasic effects of $[Ca^{2+}]i$ on agonist-

stimulated cAMP accumulation. It may be possible to isolate and purify a Ca^{2+}/calmodulin dependent protein kinase (Types I, III, VIII adenylate cyclases) to explain Tg's potentiation of agonist-stimulated cAMP accumulation, and it may also be possible to isolate and purify a Ca^{2+}-inhibitable adenylate cyclase isoform (Types V and VI adenylate cyclases) to explain the inhibition by K^+ of agonist-stimulated cAMP accumulation.

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Appendix 1

Phosphate-Buffered Sa' 'e

Sodium Chloride	140 mM
Potassium Chloride	2 mM
Sodium Phosphate Dibasic	10 mM
Potassium Phosphate Monobasic	2 mM
pH	7.4

Appendix 2

Buffer for GH RIA

Sodium Phosphate Dibasic	10 mM
Sodium Chloride	145 mM
Disodium Ethylenediamine Tetracetate	25 mM
Bovine Serum Albumin	10 g
Merthiolate	200 mg
pH	7.6

Appendix 3

Fura 2 Medium

Sodium chloride	140 mM
Potassium chloride	5 mM
Calcium chloride	2 mM
Magnesium chloride	1.2 mM
Potassium phosphate monobasic	1.2 mM
HEPES	25 mM
Glucose	6 mM
pH	7.2